Volume 45 *October 1959* *Number 10*

Proceedings of the
National Academy of Sciences
of the United States of America

The Proceedings of the National Academy of Sciences

OFFICERS OF THE ACADEMY

DETLEV W. BRONK
President

FARRINGTON DANIELS
Vice President

H. P. ROBERTSON
Foreign Secretary

HUGH L. DRYDEN
Home Secretary

WILLIAM J. ROBBINS
Treasurer

EDITORIAL BOARD OF THE PROCEEDINGS

WENDELL M. STANLEY
Chairman

HUGH L. DRYDEN, *Home Secretary*
WILLIAM J. ROBBINS, *Treasurer*
H. P. ROBERTSON, *Foreign Secretary*
EDWIN B. WILSON, *Managing Editor*

GREGORY BREIT
HARRISON BROWN
ERNST CLOOS
THOMAS FRANCIS, JR.
C. H. GRAHAM
SAUNDERS MAC LANE
H. W. MAGOUN
HARLOW SHAPLEY
PAUL WEISS

Subscription price is $12.50 for 1 year, $24.00 for 2 years, and $34.50 for 3 years; the price of a single issue is $1.50. Make all remittances payable to the National Academy of Sciences in U.S. currency or its equivalent.

Editorial correspondence should be addressed to the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Avenue, Washington 25, D. C.

Business correspondence should be addressed to the Printing and Publishing Office, National Academy of Sciences, 2101 Constitution Ave., Washington 25, D. C.

Subscribers are requested to notify the Printing and Publishing Office of the Academy and their local postmaster immediately of change of address. Notices to the Academy should provide both the old and the new address.

Microfilms of complete volumes of this journal are available to regular subscribers only and may be obtained at the end of the volume year from University Microfilms, 313 N. First Street, Ann Arbor, Michigan.

Second-class postage paid at Easton, Pennsylvania. This publication is authorized to be mailed at the special rates of postage prescribed by Section 132.122.

PRINTED IN THE U. S. A.

THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
is published monthly by
THE NATIONAL ACADEMY OF SCIENCES

Proceedings of the
NATIONAL ACADEMY OF SCIENCES

Volume 45 · Number 10 · October 15, 1959

COORDINATE REPRESSION OF THE SYNTHESIS OF FOUR HISTIDINE BIOSYNTHETIC ENZYMES BY HISTIDINE

By Bruce N. Ames and Barbara Garry

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, BETHESDA, MARYLAND

Communicated by Herman M. Kalckar, August 20, 1959

Introduction.—In several instances, the rate of synthesis of the enzymes of a biosynthetic pathway appears to be regulated by the size of the pool of the end product of that pathway.[1–4] This phenomenon has been called enzyme repression.[2]

We have observed repression by histidine during the investigation of the histidine biosynthetic enzymes in histidine-requiring mutants of *Salmonella typhimurium*.[5] The cellular concentration of the four histidine biosynthetic enzymes which were examined were increased about 15-fold by limiting the amount of histidine available to a mutant during growth. A simple method for limiting the availability of histidine in the cell during the entire growth period, and thus increasing the enzyme concentrations, was realized by growing histidine-requiring mutants on a derivative of histidine, formylhistidine, as a source of this amino acid. It appeared, therefore, that the normal low level of the biosynthetic enzymes in wild-type cells grown on minimal medium was probably due to the repression by the normal pool of histidine of most of the cellular capacity for synthesizing these proteins.

This investigation is concerned with utilizing the histidine biosynthetic system from *Salmonella* to answer the following question: Does the histidine repression of enzyme synthesis affect each of the enzymes of the pathway to the same extent, i.e., is there a parallel increase in specific activity of all four enzymes on lowering the internal histidine pool and a constant ratio of the activity of one histidine enzyme to another independent of the concentration of the enzymes in the cell? A secondary point of interest is whether there is any influence of the concentration of the enzyme substrates on the enzyme synthesis control mechanism.

The strains used in this study were some of the several hundred histidine-requiring mutants of *Salmonella* that have been mapped by Hartman, *et al.*[6, 7] They were able to demonstrate that each of the mutants could be classified into one of the seven genetic loci, *E*, *F*, *A*, *B*, *C*, *D*, and *G*, which were closely linked on the chromosome in the above sequence. These same mutants were analyzed by us for presence or absence of various enzymes of the histidine biosynthetic pathway.[5] The genetic data taken in conjunction with the biochemical analysis gave more direct support for Hartman's original suggestion[8] that the sequence of the genes in the linkage map corresponds to the sequence of the enzymes they control in the biosynthetic pathway.

The pathway of histidine synthesis seems to involve six enzymes, the last four of which have been studied in this investigation.

(1) The first enzyme catalyzes the condensation of phosphoribosyl pyrophosphate (PRPP) with AMP to give an as yet uncharacterized compound called Compound III (Moyed and Magasanik[9]). This enzyme appears to be lacking in the histidine *F* mutants in *Salmonella*.[9, 10]

(2) The second enzyme converts Compound III to imidazoleglycerol phosphate (IGP) and appears to be controlled by the *A* gene.[9, 10]

(3) The third enzyme is IGP dehydrase which catalyzes the conversion of IGP to imidazoleacetol phosphate (IAP), and is missing in mutants of the *B* class.[5, 11]

(4) The fourth enzyme, IAP transaminase, catalyzes the transamination of IAP to histidinol phosphate and is missing in mutants of the *C* class.[5, 12]

(5) The fifth enzyme histidinol phosphate phosphatase hydrolyzes histidinol phosphate to histidinol.[5, 13] No mutants have as yet been found in *Salmonella* missing only this enzyme (cf. *Discussion* in Ames, *et al.*[5]). The gene for the phosphatase does appear to be linked to the rest of the histidine genes, however, as certain multisite mutants are missing the phosphatase in addition to the other histidine enzymes. This enzymatic step appears to be experimentally irreversible.

(6) The last enzyme in the pathway is histidinol dehydrogenase which oxidizes histidinol to histidine (Adams[14]), and which is associated with the *D* gene.[5] This enzymatic step also appears to be irreversible.

Possible reasons for the histidine requirement of *E* and *G* mutants, which seem to have all these enzymes, have been briefly discussed.[5]

Methods.—The conditions for growing the various mutants, method of making extracts, and assays for the last four histidine biosynthetic enzymes have been described in detail.[5]

Glutamic dehydrogenase assay: Glutamic dehydrogenase, an enzyme involved in a different biosynthetic pathway, has been assayed as a control in some of the experiments. The reaction catalyzed by the enzyme is:

$$\text{L-glutamate} + \text{DPN} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{DPNH} + \text{NH}_3 + \text{H}^+.$$

In the assay for this enzyme the DPNH formed in the oxidation of glutamate was coupled, with diphorase, to the reduction of the blue dye dichlorophenolindophenol. The assay was performed in a Cary recording spectrophotometer at 25° by following the disappearance of the blue color of the dye at 600 mμ. The assay cuvettes contained: 0.5 ml. of 0.4 mM sodium dichlorophenolindophenol, 0.4 ml of 0.2 *M* triethanolamine-HCl buffer (pH 8.4), 0.005 ml of 50 mM TPN (brought to pH 5 with KOH), an excess of diaphorase (0.01 ml[15]), and 0.005 to 0.03 ml of dialyzed extract. After the small endogenous oxidation had ceased and the dye was not reduced further, 0.05 ml. of 0.5 M L-glutamate (brought to pH 8.5 with KOH) was added and the rate of dye bleaching determined. The oxidation of 0.01 μmole of glutamate results in a decrease in optical density of 0.3 under these conditions. One unit of enzyme activity is defined as the quantity required for the oxidation of one μmole of glutamate per hour under the assay conditions.

Results.—The enzyme levels of hisE-11 during growth on limiting histidine: A "leaky" mutant *hisE-11*, which can make some histidine and grow slowly on minimal medium[6] has been found to produce large quantities of the histidine enzymes

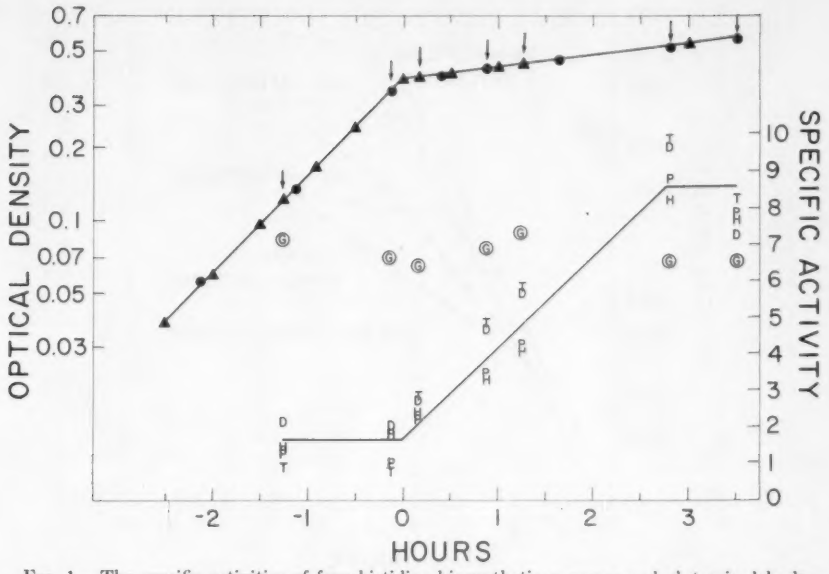


FIG. 1.—The specific activities of four histidine biosynthetic enzymes and glutamic dehydrogenase during the growth of the "leaky" histidine-requiring mutant *hisE-11*. The amount of histidine which was added (0.03 mM) was exhausted midway during the growth period (zero time) and the mutant then grew at a lower rate which was limited by the small amount of histidine it was capable of making. A liter culture was grown in a two-liter flask, shaken at 37° in a rotary shaker,[16] and the optical density readings were determined at various times. The -▲- and -●- symbols on the growth curves represent two separate experiments, run under identical conditions, which are both plotted. At the times indicated by the arrows, 100-ml aliquots of the bacterial culture were harvested, cooled rapidly, and centrifuged in the cold. Extracts were prepared from the bacteria, and protein and enzyme were assayed as previously described[5] (cf. Methods). The specific activities of the phosphatase, P, and glutamic dehydrogenase, Ⓖ, have been plotted in the units (μmoles/mg protein/hour) previously described.[5] The dehydrase, *D*, the transaminase, *T*, and the histidinol dehydrogenase, *H*, specific activities have been normalized by multiplying by the factors 1.1, 3.5, and 7.5 respectively. These factors were determined from the slopes of the lines in Figures 3, 4, and 5, and make the specific activities of each of the histidine enzymes approximately equal.

during the period of slow growth on minimal medium. The kinetics of enzyme formation were examined and are shown in Figure 1. The culture was analyzed for four histidine biosynthetic enzymes during the period of growth on histidine (0.03 mM) as well as during the period after the exhaustion of the exogenous histidine supply when the mutant was growing at its slow "leaky" rate. A control enzyme, glutamic dehydrogenase, which is involved in another amino acid biosynthetic pathway was also assayed in the extracts. Low, wild-type levels of the four histidine enzymes were found during the exponential phase of growth, when histidine was present in excess. When the histidine was exhausted and the growth rate decreased, the specific activity of the histidine enzymes in the cells started to increase rapidly. For mutant *hisE-11* the concentration of the enzymes finally approaches a level that corresponds to an eight-fold increase over that found in the wild-type strain. The kinetic data of Figure 1 indicate that, within the limits of accuracy of the assays on crude extracts, the specific activity of each of the histidine enzymes begins to increase at the same time and increases in parallel with the other histidine

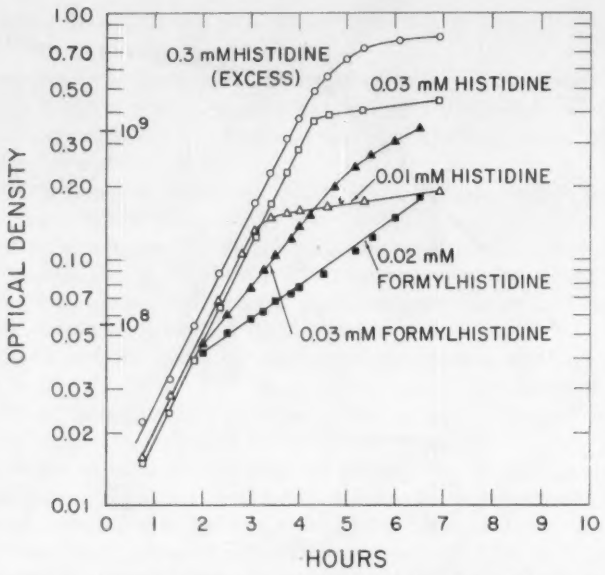


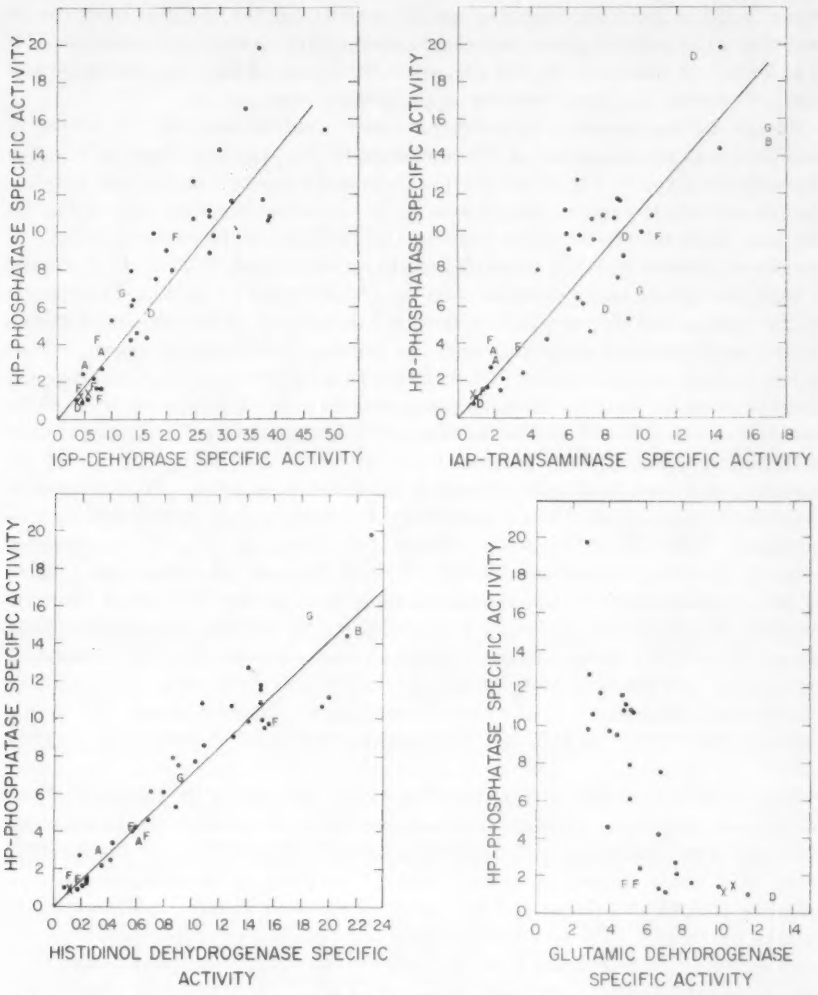
FIG. 2.—*Expt. A*—(L-histidine: Δ, 0.01 mM; □, 0.03 mM; ○, 0.3 mM). The optical density of growing cultures of mutant *hisF-41* on differing amounts of histidine were determined at various times in a Beckman DU spectrophotometer at 650 mμ. The inoculum was grown on 0.2 mM L-histidine. The culture was grown at 37° in a rotary shaker; the minimal medium used and procedures have been previously described.

Expt. B—(Nα-formyl-L-histidine: ■, 0.02 mM; ▲, 0.03 mM). The growth rates of mutant *hisF-41* on differing amounts of formylhistidine were determined in a separate experiment. The conditions were the same as in Figure 1a. Data from a histidine control culture and a culture which was 0.04 mM in formylhistidine, both of which gave the same growth rate as was obtained in Figure 1a, are not presented. No growth was obtained with a culture which was 0.01 mM in formylhistidine.

enzymes. The glutamic dehydrogenase specific activity on the other hand does not increase, but remains relatively constant.

When *hisE-11* or any of the other mutants was grown on excess histidine, the final concentration of the histidine enzymes was the same as in the wild-type cells.[5] The behavior of this mutant is contrasted with other histidine mutants which stop growing soon after histidine becomes exhausted and which do not show an increase in the specific activity of the histidine biosynthetic enzymes when examined several hours after growth has stopped.

The effect of formylhistidine on growth rate and enzyme level: The growth curves for a typical histidine-requiring mutant, *hisF-41*, grown on differing amounts of histidine is shown in Figure 2. It is apparent that the leveling off point is determined by this histidine concentration, while the growth rate is not. The histidine pool does not limit the growth rate under these conditions. The growth rate was limited by histidine deficiency, however, when the mutant was grown on a derivative of histidine, N-α-formyl-L-histidine,[17] Figure 2. The long generation time of the



FIGS. 3, 4, 5, 6.—The specific activities of the histidine enzymes and glutamic dehydrogenase in various extracts. Each point represents the specific activities of two of the enzymes in an extract of a mutant or wild-type. The wild-type extracts are symbolized by (X), the *E* class of mutants by (●) and the other classes by their letters: F, A, B, C, D, G. The growth conditions are the same as previously described[5] and as discussed in the text.

formylhistidine-grown cells was presumably the result of either slow uptake or slow hydrolysis of formylhistidine by the cells. The growth rate of the wild-type cells on 1.0 mM histidine or 0.03 mM formylhistidine was the same as that of a mutant grown on histidine.

The concentration of enzymes in the wild-type cells grown on minimal medium, a concentration which is relatively constant throughout the growth cycle, has been used as a point of reference in these studies. Mutants that have been grown on

excess histidine have the wild-type specific activity for the histidine biosynthetic enzymes, while mutants grown on formylhistidine have up to fifteen times the wild-type level.[5] A similar effect with two other derivatives of histidine, carnosine and histidylhistidine, has been observed in preliminary experiments.

Specific activity changes of the histidine enzymes in various mutants: It became of interest to examine a variety of different mutants for parallel changes in histidine biosynthetic enzymes, i.e., to see whether there was a constant ratio of one histidine enzyme activity to another independent of the concentration of the enzymes in the cell and independent of which enzyme the histidine mutant was missing. A variety of different histidine mutants from the seven classes, *E*, *F*, *A*, *B*, *C*, *D*, and *G* have been grown under different conditions with respect to the histidine content of the medium and then assayed for the last four enzymes of histidine biosynthesis as well as for glutamic dehydrogenase. In general, the changes in concentrations of the enzymes were as follows: When wild-type cells were grown on minimal medium or when the histidine mutants were grown on excess histidine low levels of the histidine enzymes were found in the extracts.[5] Extracts of *hisE-11* grown on 0.03 mM histidine (Fig. 1), and harvested several hours after the exhaustion of the histidine, contained intermediate levels of the histidine enzymes. High concentrations of the enzymes were found in extracts of mutants grown on 0.03 mM formylhistidine.[5] The ratio of the specific activity of the dehydrase (Fig. 3), transaminase (Fig. 4), histidinol dehydrogenase (Fig. 5), and glutamic dehydrogenase (Fig. 6) to the specific activity of the phosphatase has been plotted for each of these extracts.[18] In cases where a mutant was missing one of the four enzymes examined, the specific activity of that enzyme was not plotted. Figures 3, 4, and 5 show that the ratio of one histidine biosynthetic enzyme activity to another is constant no matter what the mutant or the growth conditions. Figure 6 shows that such a relation is not true for the glutamic dehydrogenase-histidinol phosphate phosphatase ratio.

Separation of the histidine biosynthetic enzymes: Because of the constant ratio of one enzyme activity to another, it was of interest to see whether the four enzyme activities were associated with separate proteins. Figure 7 shows the fractionation of a crude extract of mutant *hisE-11* obtained on a diethylaminoethylcellulose (DEAE)[19] column and the assay of the four enzyme activities in the column fractions.[20] The IAP-transaminase and histidinol dehydrogenase activities were eluted as discrete peaks while the histidinol phosphate phosphatase and IGP-dehydrase activities came off together in a third peak. It is not clear whether this association of the phosphatase and dehydrase is fortuitous or represents some actual physical association. The fact that the four enzymes are associated with a minimum of three distinct proteins does indicate, however, that the parallel enzyme changes cannot be explained by the association of all the enzyme activities on the same protein molecule.

Discussion.—The data presented indicate that in acting as a repressor, histidine appears to affect the synthesis of each of the histidine biosynthetic enzymes to the same extent, within the experimental error of the assays. It is proposed that this phenomenon be called coordinate repression.

An indication that the histidine repression is independent of the quantity of the substrate of each enzyme present in the cells (cf. also Yates and Pardee[3]) is given by

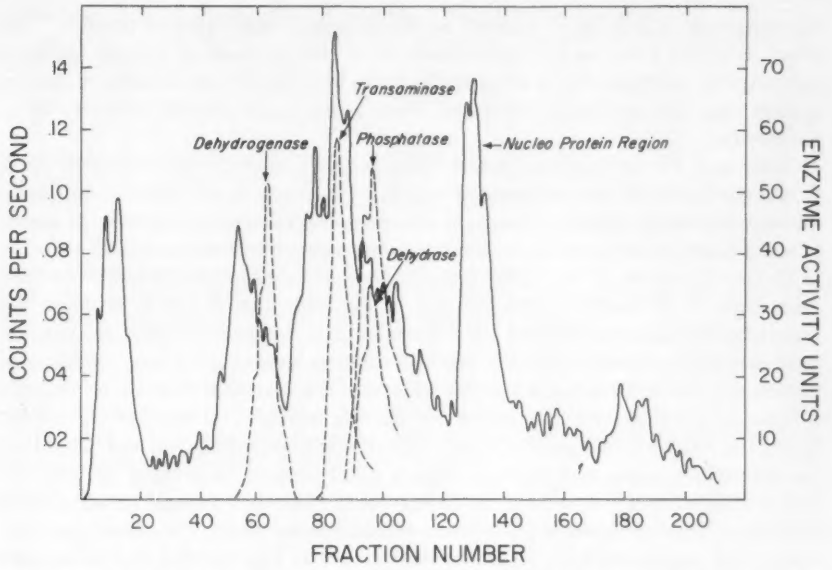


FIG. 7.—The fractionation on a DEAE column of an extract of mutant *hisE-11* grown on formylhistidine. The bacteria were grown as previously described,[5] except that randomly labeled C^{14}-fructose was used as a carbon source instead of glucose. The bacteria were broken in a French pressure cell (cf. Cowie *et al.*[21]). 35 mg. of protein were run on the column[21] by Dr. Dean Cowie who also analyzed the eluate fractions for radioactivity and protein.[22] The protein and radioactivity determinations showed a constant ratio and only the latter has been plotted (——). The activities of the four histidine enzymes(– – –) were determined as previously described.[5] The dehydrogenase units have been multiplied by a factor of 10 and the dehydrase units by a factor of 2 so that the peaks are of approximately the same height. Enzymes were localized by assaying combined aliquots from groups of ten tubes; no enzyme activity was detected for any particular enzyme in fractions other than the peak indicated. Over 90 per cent of the activity of each enzyme put on the column was recovered in the combined fractions from each peak. The specific activities of the enzymes in the crude extract were the same as previously found for mutant *hisE-11* grown on formylhistidine.[5] The specific activities of the pooled fractions for each enzyme represented a five- to 10-fold increase over the crude extract.

the finding that mutants from all genetic classes show these coordinate changes in enzyme levels. It had been previously shown that mutants accumulate intermediates before the enzymatic block in large amounts[6, 8] and since the last steps of the pathway appear to be irreversible the mutants have presumably never contained the histidine precursors after the enzymatic block. Thus, depending on the mutant, an enzyme can be in the presence of no substrate or an abnormally large amount of substrate without influencing the repression of its synthesis by histidine.

Many hypotheses would be consistent with the finding that histidine represses the synthesis of all of the biosynthetic enzymes together. One attractive possibility is that the repressor blocks enzyme synthesis at the gene level.[23] The genes for the series of histidine enzymes have been shown by Hartman[6, 8] to be closely linked on the *Salmonella* chromosome and conceivably this length of the chromosome is "functionally turned off" by a histidine-nucleic acid repressor (e.g., histidine-soluble RNA) with a specific affinity for part of the histidine section of the chromosome. In *Neurospora*[24] and yeast,[25] where the histidine genes are scattered on the

chromosomes, it will be of interest to see if enzyme repression is possible. No effect of added histidine has been observed on the synthesis of various histidine biosynthetic enzymes in *Neurospora*.[26] However, this is not conclusive as the system may be maximally repressed when growing on minimal medium as is *Salmonella*.

Yates and Pardee[3] have examined three enzymes of orotic acid biosynthesis in *E. coli*, each of which was repressed by uracil. Their data do not indicate coordinate repression as each enzyme appears to change independently in response to uracil. It is not known whether the genes for these enzymes are linked or not in *E. coli*.

The use in nature of this latent capacity to make histidine enzymes in wild-type *Salmonella* is of interest. The function of the repression is not to decrease the amount of histidine synthesized when histidine is in the medium, since the enzyme levels are not repressed below the minimal medium level even by large amounts of histidine. The work of Yates and Pardee[3] and of Schwartz *et al.*,[27] however, suggests a function for this cellular capacity for making increased biosynthetic enzymes. Yates and Pardee investigated a uracil biosynthetic enzyme in *E. coli* and found that on a complete amino acid medium (which could support very rapid growth) the cells contained over five times as much enzyme as when they were grown on minimal medium. This increased synthesis was repressible by uracil. A similar case has been found in arginine biosynthesis by Schwartz *et al.* who found a six-fold increase in ornithine transcarbamylase in *E. coli* grown on an arginine-free enriched medium as compared to a minimal medium. Thus wild-type bacteria which are capable of growing with a doubling time of close to 15 min. in a highly enriched medium without histidine would presumably need much more of the histidine enzymes under these conditions than they would when growing on minimal medium with a doubling time of close to 40 min. Certain systems cannot be repressed below the minimal medium enzyme level (e.g., histidine, uracil[3]) while others can (e.g., arginine [4, 27]).

Another mechanism, distinct from enzyme repression, appears to regulate histidine synthesis at a substrate level when excess histidine is present in *Salmonella*. This mechanism, the inhibitory effect of the end product of a pathway on the activity of an early enzyme of the pathway, has been called "feedback control" and has been described by a number of investigators[28–34] in a variety of organisms. Moyed and Friedman[35] have presented some evidence that in *E. coli* histidine inhibits the activity of the first enzyme in the biosynthetic pathway and thus presumably prevents the synthesis of histidine when excess histidine is present in the medium. A feedback control in both *Salmonella* and *Neurospora* is indicated by the fact that histidine precursors do not accumulate before a genetic block when excess histidine is added to the minimal medium[26] through the specific activity of the histidine biosynthetic enzymes are not lowered.

[1] Cohn, M., G. N. Cohen, and J. Monod, *Compt. Rend.*, **236**, 746 (1953).

[2] Vogel, H. J., in *The Chemical Basis of Heredity*, ed. McElroy and Glass (Baltimore: Johns Hopkins Press, 1957), p. 276.

[3] Yates, R. A., and A. B. Pardee, *J. Biol. Chem.*, **227**, 677 (1957).

[4] Gorini, L., and W. K. Maas, *Biochim. et Biophys. Acta*, **25**, 208 (1957).

[5] Ames, B. N., B. Garry, and L. A. Herzenberg, *J. Gen. Microbiol.* (in press).

[6] Hartman, P. E., J. C. Loper, and D. Šerman, *J. Gen. Microbiol.* (in press). We wish to thank Dr. P. E. Hartman for supplying these mutants and for many helpful discussions concerning this investigation.

[7] Hartman, P. E., Z. Hartman, and D. Šerman, *J. Gen. Microbiol.* (in press).

[8] Hartman, P. E., in *Genetic Studies with Bacteria*, Carnegie Inst. of Wash. Publ. **612** (Washington, D.C., 1956), p. 35.

[9] Moyed, H. S., and B. Magasanik, *J. Am. Chem. Soc.*, **79**, 4812 (1957).

[10] Moyed, H. S., personal communication.

[11] Ames, B. N., *J. Biol. Chem.*, **228**, 131 (1957).

[12] Ames, B. N., and B. L. Horecker, *J. Biol. Chem.*, **220**, 113 (1956).

[13] Ames, B. N., *J. Biol. Chem.*, **226**, 583 (1957).

[14] Adams, E., *J. Biol. Chem.*, **209**, 829 (1954); **217**, 325 (1955).

[15] Worthington Biochemical Corp., Freehold, New Jersey. The protein concentration of this diaphorase solution is approximately 5 mg/ml.

[16] New Brunswick Scientific Co., New Brunswick, New Jersey.

[17] Fischer, E., and L. H. Cone, *Annalen*, **363**, 116 (1908). We are indebted to Dr. Howard Bond who synthesized some of the formylhistidine used in this study.

[18] In the dialysis of the extracts in some of the later experiments tris-(hydroxyamino)methane buffer, 0.01 *M*, pH 7.5, was substituted for the triethanolamine buffer, 0.01 *M*, pH 7.5, described previously[4] as this buffer did not require a correction in the protein determinations.

[19] Peterson, E. A., and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956).

[20] This experiment was done in collaboration with Dr. Dean Cowie to whom we are indebted for permission to present these data and for helpful discussions.

[21] Cowie, D. B., G. N. Cohen, E. T. Bolton, and H. de Robichon-Szulmajster, *Biochim. et Biophys. Acta*, **34**, 39 (1959).

[22] Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1953).

[23] It has been called to our attention that a similar hypothesis has been suggested by Dr. F. Jacob, Harvey Lecture (1959), in press.

[24] Haas, F., M. B. Mitchell, B. N. Ames, and H. K. Mitchell, *Genetics*, **37**, 217 (1952).

[25] Leupold, U., *Cold Spring Harbor Symposium Quant. Biol.*, **23**, 161 (1958).

[26] Ames, B. N., unpublished observations.

[27] Schwartz, J. H., W. K. Maas, and E. J. Simon, *Biochem. et Biophys. Acta*. **32**, 582 (1959).

[28] Adelberg, E. A., and H. E. Umbarger, *J. Biol. Chem.*, **205**, 475 (1953).

[29] Brooke, M. S., D. Ushiba, and B. Magasanik, *J. Bact.*, **68**, 534 (1954).

[30] Yates, R. A., and A. B. Pardee, *J. Biol. Chem.*, **221**, 757 (1956).

[31] Strecher, H. J., *J. Biol. Chem.*, **225**, 825 (1957).

[32] Wormser, E. H., and A. B. Pardee, *Arch. Biochem. Biophys.*, **78**, 416 (1958).

[33] Umbarger, H. E., and B. Brown, *J. Biol. Chem.*, **233**, 415 (1958).

[34] Wyngaarden, J. B., and D. M. Ashton, *Nature*, **183**, 747 (1959).

[35] Moyed, H. S., and M. Friedman, *Science*, **129**, 968 (1959).

---

*AMINO ACID ACTIVATION AND TRANSFER TO RIBONUCLEIC ACIDS IN THE CELL NUCLEUS**

BY J. W. HOPKINS

THE ROCKEFELLER INSTITUTE

Communicated by Alfred E. Mirsky, August 14, 1959

The *in vitro* incorporation of amino acids into the proteins of calf thymus nuclei (isolated in isotonic sucrose) has been shown to be dependent upon the presence of ATP.[1, 2] In isolated nuclei ATP synthesis depends on an aerobic phosphorylating system which differs from that of mitochondria in being insensitive to various mitochondrial inhibitors (carbon monoxide, methylene blue, and calcium ions).

All agents which have been found to block nuclear generation of ATP also block amino acid incorporation into proteins.[1, 2]

Zamecnik and his co-workers[3, 4] and Siekevitz[5] have described energy dependent, cell-free cytoplasmic systems which incorporate amino acids into protein. The first step in the process is considered to be an enzymatic activation of the carboxyl groups of amino acids.[6] This reaction requires ATP. The amino acyladenylate compounds which are formed remain bound to the enzyme surface.[3]

$$\text{ATP} + \text{AA} + \text{Enzyme} = \text{Enzyme}-\text{AMP}-\text{AA} + \text{PP}$$

Subsequently the activated amino acid is transferred to the 2′ or 3′ position of the terminal adenine nucleotide of soluble ribonucleic acids by the activating enzyme.[7, 8, 4]

$$\text{E}-\text{AMP}-\text{AA} + \text{sRNA} = \text{sRNA}-\text{AA} + \text{AMP} + \text{E}$$

Enzymes catalyzing these reactions, the pH 5 enzymes, have been prepared from extracts of a variety of animal,[3, 9] plant, [10, 12] and bacterial cells[13, 14] by precipitation (from the soluble components of the cell) at pH 5. In cytoplasmic protein synthesis, incorporation of the amino acid into polypeptides takes place later, and requires the presence of the microsomal fraction.[15]

The present paper is concerned with a similar series of reactions in cell nuclei.

Evidence will be presented to show that pH 5 activating enzymes are present in significant amounts in cell nuclei prepared in both aqueous and nonaqueous media. During the process of incorporation of radioactive amino acids by isolated calf thymus nuclei, a part of the nuclear RNA (which can be isolated by the phenol method[16]) becomes labeled with the amino acid.

Materials and Methods.—Fresh calf thymus nuclei were prepared in isotonic sucrose with 0.0030 *M* $CaCl_2$ by the method previously described.[1] After washing twice with sucrose solution, pH 5 enzymes were prepared from the nuclear pellet following the procedure used by Hoagland *et al.* for rat liver.[3] The precipitate formed at pH 5.2 was dissolved in 0.1 *M* tris buffer at pH 7.6. In most preparations this solution was dialyzed against 2 changes of 100 volumes each of this buffer for 3 hr to remove free amino acids. (All steps were carried out at 0° to 2°C.) The pH 5 enzymes from whole thymus tissue were prepared similarly using well minced tissue rather than the nuclear pellet as starting material.

Nonaqueous nuclei from calf thymus and chicken kidney and their tissue "controls" were isolated as described by Allfrey *et al.*[17] The pH 5 enzymes from the nonaqueous material were prepared and assayed in the same way as described for fresh material.

Amino acid-dependent exchange of P^{32}-pyrophosphate with ATP was used to determine the amino acid activating activity of the pH 5 enzyme fraction. P^{32}-pyrophosphate was prepared by pyrolysis. The ATP^{32} formed was adsorbed on charcoal and its radioactivity was determined by the procedure of Crane and Lipmann.[18] Phosphorus analyses were done using Allen's method.[19] Protein concentration was measured by the method of Lowry[20] or by direct weighing.

Two μmoles each of the 12 L-amino acids used by Hoagland *et al.*[3] were employed for most assays. These were leucine, isoleucine, valine, glycine, threonine, histidine, phenylalanine, tryptophan, serine, alanine, arginine, and lysine. DNAase and RNAase were supplied by Worthington Biochemical Corp.; Na_2ATP was

from Sigma Chemical Co.; Parke and Davis Chloramphenicol was used. The pH of all solutions was adjusted to 7.6 before use.

RNA was prepared by the phenol method of Kirby[16] using freshly distilled phenol. The RNA was washed 4 times with cold 2 per cent perchloric acid, 3:1 ethanol:

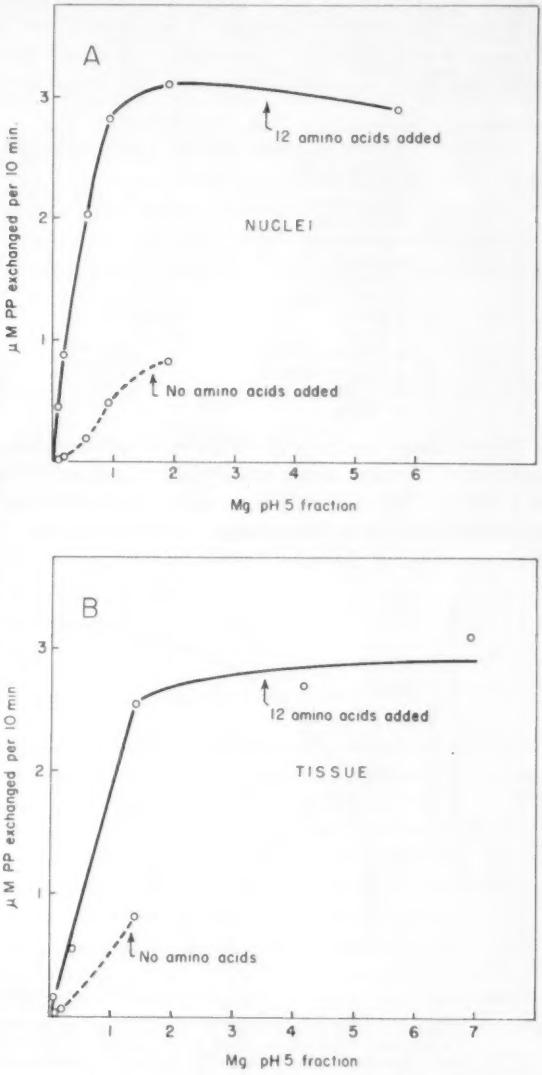


FIG. 1.—The relationship between ATP P^{32}-pyrophosphate exchange and concentration of the pH 5 enzyme fraction from fresh calf thymus nuclei (A) and tissue (B).

Each flask contained 5 μmoles of ATP, 1 μmole of P^{32}-pyrophosphate and the indicated amounts of pH 5 enzymes in 0.1 *M* "tris" buffer at pH 7.6. Two μmoles each of the 12 L-amino acids listed in the text were added. The solution contained 5 μmoles $MgCl_2$ and 10 μmoles KF. The final volume was 1.0 ml. Incubations were carried out in air at 37°C with gentle agitation for 10 min. The exchange reaction was stopped by adding 2.0 ml cold 10 per cent trichloroacetic acid.

ether, and ether to insure complete removal of free amino acids before determining its radioactivity. Protein was prepared for counting as described by Allfrey *et al.*[1] Radioactivity was measured in a thin-window gas flow counter and the counts were corrected for self absorption by the method of Schweitzer and Stein.[21]

Results.—Extracts of thymus nuclei and whole tissue catalyze the ATP P^{32}-pyrophosphate exchange reaction. Figure 1 illustrates the amino acid-dependent nature of the exchange. When the pH 5 enzyme fraction is prepared as described, but not dialyzed, some exchange is observed in the absence of added amino acids. This is due presumably to the presence of residual free amino acids. Dialysis for 3 hr reduces this residual exchange to less than 15 per cent of that observed in the presence of a mixture of 12 amino acids. A similar effect can be obtained by treatment with Norit A, following the procedure described by Clark.[12]

TABLE 1

EFFECT OF AMINO ACIDS ON PYROPHOSPHATE-ATP EXCHANGE

Amino Acids Promoting Exchange	Amino Acids Without Effect in This System
L-Cysteine	D-Amino acids
L-Histidine	L-Alanine
L-Isoleucine	L-Arginine
L-Lysine	L-Aspartic acid
L-Leucine	L-Glutamic acid
L-Methionine	Glycine
L-Proline	L-Phenylalanine
L-Serine	
L-Threonine	
L-Tryptophan	
L-Tyrosine	
L-Valine	

The exchange occurs with mixtures of amino acids or with amino acids tested separately. Twelve amino acids which promote PP^{32} are listed in the first column of Table 1. The nuclear pH 5 fraction from fresh calf thymus does not promote exchange reactions in the presence of D-amino acids. To date, no stimulation has been observed for the other 6 amino acids listed in Table 1.

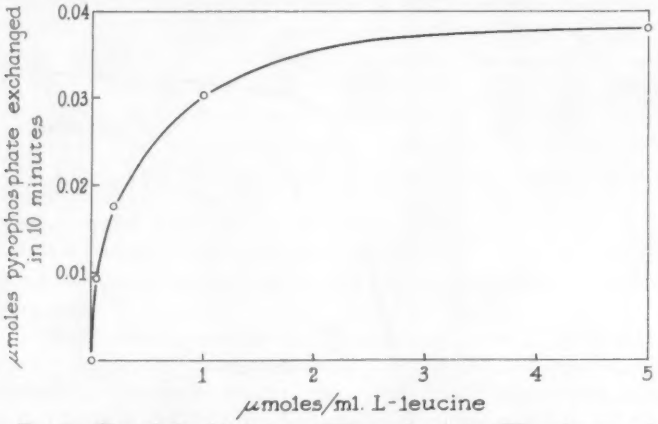


FIG. 2.—The relationship between P^{32}-pyrophosphate-ATP exchange and the amount of L-leucine added to the pH 5 enzyme fraction of calf thymus nuclei. Incubation conditions were the same as described in the legend to Figure 1. One milligram pH 5 fraction was added.

The rate of exchange depends on amino acid concentration. Figure 2 illustrates this dependence for L-leucine.

The evidence for nuclear localization: The occurrence of amino acid activating

enzymes in nuclei isolated in *aqueous* media is not in itself a proof of nuclear localization. Since these enzymes are water-soluble, the possibility must be considered that their presence in nuclei is the result of adsorption of cytoplasmic enzymes during the process of isolation.

The transfer or exchange of enzymes between nucleus and cytoplasm in the course of isolation in *aqueous media* is well documented.[22] However, a proof of nuclear localization can be obtained from a study of nuclei isolated in *nonaqueous media* by the Behrens procedure[23] or its modifications.[24, 17] In this method the tissue is rapidly frozen and lyophilized. This prevents any loss or exchange of soluble cell components. After grinding, the nuclei are isolated in mixtures of aqueous solvents by density gradient centrifugation.

Nuclear preparations of high purity can be obtained. Particularly pure preparations have been isolated from chicken kidney and calf thymus.[25]

These preparations have been tested for their content of amino acid activating enzymes. The results are summarized in Table 2. For purposes of comparison,

TABLE 2

	Column 1 Activity* per mg pH 5 Fraction	Column 2 Activity* per 100 mg Starting Material	Column 3 Activity* per 100 mg Whole Cells		Column 4 Per Cent of Total Activity in the Nucleus
			Nucleus	Cells	
Behrens calf thymus nuclei	0.050	0.082	0.050	...	70.4%
Behrens calf thymus tissue	0.048	0.071	...	0.071	...
Behrens chicken kidney nuclei	0.051	0.120	0.018	...	7.6%
Behrens chicken kidney tissue	0.080	0.236	...	0.236	...
Fresh calf thymus nuclei	0.46	0.570	0.348	...	62.5%
Fresh calf thymus tissue	0.22	0.557	...	0.557	...

* μmoles pyrophosphate exchanged in 10 min. Incubation conditions as in Figure 1.

the activities oberved in extracts of isolated nuclei have been compared with those of whole tissue extracts. (Tissue "controls" were prepared by lyophilizing and treating with the same organic solvents used in the isolation of nuclei.[17]) The pH 5 enzymes were prepared from nuclei and whole tissue as described in *Methods*.

The first column in Table 2 lists the specific activities of the activating enzyme fractions (as μmoles of pyrophosphate exchanged per 10 min per mg of pH 5 fraction). A mixture of 12 amino acids (see *Methods*) was present during the assay.

The second column indicates the total exchange activity contained in the pH 5 fraction from 100 mg starting material (either isolated nuclei or whole tissue). It can be seen that 100 mg of thymus nuclei contain slightly more activating enzyme than the equivalent weight of whole tissue. In kidney nuclei the total nuclear activity is about 51 per cent of the activity of the tissue control.

What per cent of the amino acid activating activity of the cell occurs in the nucleus?

The answer to this question can be obtained by comparing the activity of an equal number of nuclei and whole cells. Since in the thymus the nucleus comprises

61 per cent of the mass of the cell,[17] the figures for total activity of the nuclei listed in column 2 of the table can be multiplied by the factor 61/100 to give the total enzymatic activity in the nuclei of 100 mg of cells. The ratio of this figure to the activity in 100 mg of whole cells represents the fraction of the total amino acid activating activity of the whole cell that is localized in the nucleus. These ratios are given in column 4 of the table. In thymus the nuclei contain about 70 per cent of the total activity of the cell; in kidney the nuclei comprise only 15 per cent of the mass of the cell[17] and contain only about 8 per cent of the total activity.

It should be stressed, however, that other tests for the purity of chicken kidney nuclei isolated in nonaqueous media indicate that cytoplasmic contamination cannot account for the presence of the amino acid activating enzymes in the isolated nucleus. For example, the soluble enzyme, catalase, is completely absent from such nuclei although it is abundant in the cytoplasm. Less than 1 per cent of the total arginase activity of the cell occurs in the kidney nuclei. This is a significant test for purity because arginase occurs in extremely high concentrations in the cytoplasm of chicken kidney cells.[25]

In calf thymus nuclei isolated in nonaqueous media the presence of 70 per cent of the total activity of the cell in the nuclei cannot be explained in terms of cytoplasmic contamination. The purity of these nuclei has been established in many ways[26, 33] ranging from electron microscopy to immunological tests for purity.

The occurrence of amino acid activating enzymes in calf thymus nuclei isolated in isotonic sucrose solutions is another instance of the capacity of these nuclei to withstand isolation in this aqueous medium without a loss of water-soluble components.[22]

The specific activity of the pH 5 fraction from thymus "sucrose" nuclei is also listed in Table 2. A comparison of the total nuclear activity with the total activity of the cell shows that about 63 per cent of the amino acid activating activity of the cell is found in the nucleus. This is in good agreement with the results on nuclei isolated in nonaqueous media.

Is amino acid transferred to DNA in the nucleus? Incorporation of amino acids by calf thymus nuclei is blocked by removal of the DNA by DNAase.[1] Amino acid uptake can be restored by adding back DNA, but even RNA or synthetic polyanions (such as polyethylene sulfonate) can restore C^{14} uptake.[2] This makes it unlikely that DNA is directly involved in protein synthesis as an intermediate acceptor of activated amino acids in the nucleus. However, to test this possibility thymus nuclei isolated in 0.25 *M* sucrose were incubated with DL-leucine-2-C^{14} as described previously.[1] The nuclei were washed 3 times at 2°C with 0.9 per cent NaCl containing 0.01 *M* sodium citrate and the DNA isolated by the sodium dodecyl sulfate method B of Marko and Butler.[27] Parallel incubations were run to determine the amino acid incorporation into the total proteins of the nuclei. After 60 minutes incubation at 37°C the protein contained 381 cpm/mg while the DNA contained only 11 cpm/mg. Protein analysis of the DNA[(20)] showed 2 per cent contamination by protein. It therefore seems likely that contaminating protein can account for all, or nearly all, of the counts associated with DNA, and there is thus no evidence for a transfer of leucine-C^{14} to the DNA of the nucleus.

The inhibition of nuclear protein synthesis by chloramphenicol: Breitman and Webster[28] have shown that a high concentration (0.0067 *M*) of the antibiotic

chloramphenicol blocks amino acid incorporation in isolated calf thymus nuclei. We have confirmed this and have also tested to see whether ATP synthesis in nuclei is impaired by chloramphenicol. A comparison of ATP synthesis with and without chloramphenicol present showed that no inhibition occurred; on the contrary, slightly more ATP was present in nuclei exposed to the antibiotic.

Since chloramphenicol does not affect ATP synthesis, the question arises of its effects on amino acid activation. In a study of tryptophan activating enzyme[29] Acs and Lipmann found no effect of chloramphenicol on pyrophosphate-ATP exchange.[30] The nuclear pH 5 fraction is similarly unaffected. Furthermore, the results described below indicate that chloramphenicol does not block amino acid transfer to the RNA of isolated thymus nuclei. This indicates that the antibiotic acts at a later stage of protein synthesis.

Transfer of activated C^{14} amino acids to nuclear RNA: To test the possible involvement of nuclear RNA in protein synthesis within the nucleus, isolated calf thymus nuclei were incubated in the medium previously described (Table 3) in

TABLE 3

LABELING OF NUCLEAR RNA BY AMINO ACID

	CPM/mg
DL-Leucine-1-C^{14} (1.09 mcuries/mmole)	
Protein:	
Control	430
0.0067 *M* chloramphenicol	6
RNA:	
Control	153
0.0067 *M* chloramphenicol	161
DL-Leucine-1-C^{14} (1.09 mcuries/mmole)	
Protein:	
Control	248
0.0067 *M* chloramphenicol	8
RNA:	
Control	83
0.0067 *M* chloramphenicol	78
DL-Alanine-1-C^{14} (1.92 mcuries/mmole)	
RNA:	
Control	51
0.0067 *M* chloramphenicol	48

Incubation was at 37°C under previously described conditions.[1] After 30 min the nuclear RNA was isolated as described in the text.

the presence of DL-leucine-1-C^{14} or DL-alanine-1-C^{14}. Chloramphenicol was added to some of the flasks to prevent protein synthesis. This procedure permitted the isolation of RNA with no danger of contamination by radioactive protein. After 30 min the nuclei were centrifuged down, the medium soluble material discarded, the nuclei washed twice with 20 volumes of cold incubation mixture containing a 10-fold concentration of the C^{12}-amino acid used and the RNA isolated by the phenol method.[16] RNA was finally precipitated with ethanol, washed four times with cold 2 per cent perchloric acid, with 3:1 ethanol:ether, and with ether and counted. The extent of incorporation into the nuclear proteins, both in the presence and absence of chloramphenicol is shown in Table 3. The table also shows the incorporation of labeled amino acid into RNA. It can be seen that although 0.0067 *M* chloramphenicol inhibits nearly completely the incorporation of amino acid into protein, it has no such effect on the incorporation into RNA.

The linkage between the isolated ribonucleic acid and bound amino acid is

stable in acid and labile in alkali. Upon hydrolysis of the leucine-RNA in 0.005 *N* NaOH and subsequent chromatography on paper[31] the label is recovered as leucine. Digestion with ribonuclease also separates the radioactivity from the acid precipitable fragments. Current work is in progress to determine whether the amino acid is bound to adenylic acid or to other nucleotides of the RNAase digest.

It is a matter of some interest to know whether the RNA "carriers" in the cell nucleus are large molecules. In preliminary experiments with C^{14} leucine labeled RNA, there have been indications that some of the RNA carrying amino acid is dialyzable. This RNA is still precipitable with perchloric acid. Whether this small RNA occurs in the nucleus as such, or whether it is the result of autolysis during incubation, remains to be determined.

The time course of amino acid uptake into the RNA of the nucleus is given in Figure 3. The upper curve shows the incorporation of leucine-1-C^{14} into total

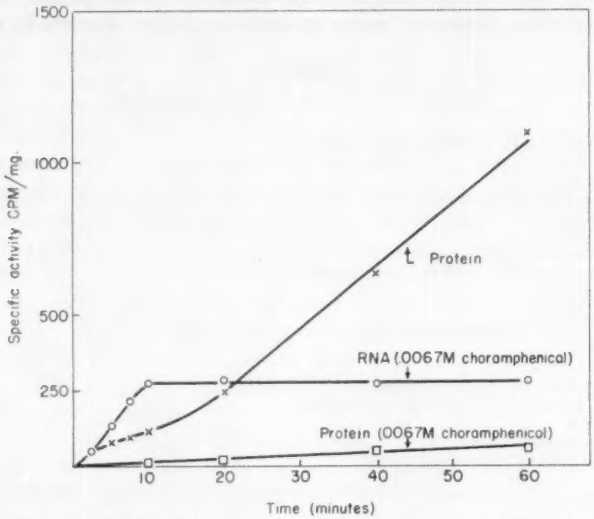


FIG. 3.—The uptake of leucine-1-C^{14} (5.3 mcuries/mmole) into RNA and protein of calf thymus nuclei. Incubation procedures were as previously described[1].

nuclear protein. This curve shows the characteristic lag period observed previously[1]. The middle curve gives the time course of leucine uptake into the isolated nuclear RNA. Chloramphenicol was present in these experiments and under these conditions the uptake into protein is negligible, as shown by the bottom curve in the figure. Although there is a lag in amino acid incorporation into protein, there is no corresponding delay in amino acid uptake into the RNA of the nucleus; this is linear up to 10 min.

These experiments suggest a direct role of nuclear RNA in protein synthesis in the nucleus. This is in accord with earlier results relating RNA to amino acid uptake by thymus nuclei where it was shown that the inhibition of RNA synthesis by the antimetabolite DRB (5,6-dichloro-β-D-ribofuranosylbenzimidazole) results in a subsequent inhibition of amino acid uptake.[1,2]

Which of the ribonucleic acids of the thymus nucleus function as carriers? This has been tested by fractionation of the nuclei after incubation with radioactive amino acids according to Allfrey *et al.*[1] The subsequent isolation of the RNA from the different fractions shows that the readily extractable RNA of the nucleus is most highly labeled with C^{14}-leucine. It is significant that treatment of isolated thymus nuclei with ribonuclease has no effect on protein synthesis[1] and also has no effect on the readily extractable nuclear RNA (while it is in the nucleus).[32] The insoluble "nucleolar" RNA fraction does not appear to function as an amino acid carrier. (It should be mentioned, however, that the isolation of the "nucleolar" RNA requires more time than the preparation of the "soluble" RNA fraction, and some loss of C^{14}-amino acid from "nucleolar" RNA might have occurred.)

It remains to be seen whether other ribonucleic acid fractions in the nucleus participate in amino acid transfer reactions.

Summary.—A pH 5 enzyme fraction catalyzing an amino acid-dependent exchange of pyrophosphate with ATP (a test for amino acid activation) has been found in calf thymus nuclei prepared in both sucrose solutions and in nonaqueous media. Similar activating enzymes occur in chicken kidney nuclei prepared in nonaqueous media.

Twelve L-amino acids have been shown to stimulate pyrophosphate-ATP exchange in the presence of the "pH 5 fraction" from calf thymus nuclei while D-amino acids have no such effect.

DNA isolated from calf thymus nuclei which have been incubated with DL-leucine-2-C^{14} and are actively incorporating amino acids into their proteins contains a negligible amount of labeled amino acid.

RNA isolated from calf thymus nuclei incubated under the same conditions contains a significant amount of labeled amino acid which is rapidly released by dilute alkali or RNAase but not by acid.

Chloramphenicol (0.0067 *M*) inhibits nearly completely the incorporation of amino acid into protein of calf thymus nuclei but has no effect on its incorporation into nuclear RNA.

The author wishes to thank Dr. V. G. Allfrey and Dr. A. E. Mirsky, in whose laboratory the work was done, for their valuable discussions and assistance.

* This research was supported in part by a grant (RG 4919) from the U. S. Public Health Service.

[1] Allfrey, V. G., A. E. Mirsky, and S. Osawa, *J. General Physiology*, **40**, 451 (1957).

[2] Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, **43**, 589 (1957).

[3] Hoagland, M. B., E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345 (1956).

[4] Hecht, L. I., M. L. Stephenson, and P. C. Zamecnik, these PROCEEDINGS, **45**, 505 (1959).

[5] Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952).

[6] Hoagland, M. B., *Biochem. Biophys. Acta*, **16**, 288 (1955).

[7] Zachau, H. G., G. Acs, and F. Lipmann, these PROCEEDINGS, **44**, 885 (1958).

[8] Preiss, J., P. Berg, E. J. Ofengand, F. H. Bergman, and M. Dieckmann, these PROCEEDINGS, **45**, 319 (1959).

[9] Lipmann, F., these PROCEEDINGS, **44**, 67 (1958).

[10] Webster, G. C., *J. Biol. Chem.*, **229**, 535 (1957).

[11] Davis, J. W., and G. D. Novelli, *Arch. Biochem. Biophys.*, **75**, 299 (1958).

[12] Clark, J. M., Jr., *J. Biol. Chem.*, **233**, 421 (1958).

[13] DeMoss, J. A., and G. D. Novelli, *Biochem. Biophys. Acta*, **22**, 49 (1956).

[14] Nisman, B., F. H. Bergman, and P. Berg, *Biochem. Biophys. Acta*, **26**, 639 (1957).
[15] Littlefield, J. W., E. B. Keller, J. Gross, and P. C. Zamecnik, *J. Biol. Chem.*, **217**, 111 (1955).
[16] Kirby, K. S., *Biochem. J.*, **64**, 405 (1956).
[17] Allfrey, V. G., H. Stern, A. E. Mirsky, and H. Saetren, *J. General Physiology*, **35**, 529 (1952).
[18] Crane, R. K., and F. Lipmann, *J. Biol. Chem.*, **201**, 235 (1953).
[19] Allen, R. J. L., *Biochem. J.*, **34**, 858 (1940).
[20] Lowry, O. H., J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193** 265 (1951).
[21] Schweitzer, G. K., and B. R. Stein, *Nucleonics*, **7**, 65 (1950).
[22] Allfrey, V. G., in *The Cell*, ed. J. Brachet, and A. E. Mirsky, (Academic Press, New York, 1959) vol. **1**, p. 193.
[23] Behrens, M., *Z. Physiol. Chem.*, **209**, 59 (1932).
[24] Dounce, A. L., G. H. Tischkoff, S. R., Barnett, and R. M. Freer, *J. General Physiology*, **33**, 629 (1949).
[25] Stern, H., V. G. Allfrey, A. E. Mirsky, and H. Saetren, *J. General Physiology*, **35**, 559 (1952).
[26] Allfrey, V. G., and A. E. Mirsky, *Science*, **121**, 879 (1955).
[27] Marko, A. M., and G. C. Butler, *J. Biol. Chem.*, **190**, 65 (1951).
[28] Breitman, T., and G. C. Webster, *Biochem. Biophys. Acta*, **27**, 409 (1958).
[29] Davie, E. W., V. V. Konigsberger, and F. Lipmann, *Arch. Biochem. Biophys.*, **65**, 21 (1956).
[30] Acs, G., and F. Lipmann, unpublished experiments.
[31] Levy, A. L., Nature, **174**, 126 (1954.)
[32] Allfrey, V. G., and A. E. Mirsky, unpublished experiments.
[33] Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, **45**, 1325(1959).

---

*A GENERAL METHOD FOR THE LABELING OF THE ACTIVE SITE OF ANTIBODIES AND ENZYMES**

BY MARIAN ELLIOTT KOSHLAND, FRIEDA ENGLBERGER, AND D. E. KOSHLAND, JR.

BIOLOGY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY

Communicated by Donald D. Van Slyke, August 19, 1959

Successful labeling of the active site has been dependent upon the particular characteristics of the enzyme system under study. One example is phosphoglucomutase which forms a kinetically stable intermediate.[1-3] Other examples are the esterases, i.e., chymotrypsin and trypsin, in which the serine at the active site reacts much more rapidly with diisopropylfluorophosphate than the other serines in the molecule.[4-8] However, there are many enzymes and proteins of special interest, such as antibodies, whose active sites do not possess any such fortunate chemical properties. In this paper a general method is presented for the labeling of the active site in these cases.

The method was developed from the classic experiments of Hopkins *et al.*[9] and the more recent developments of Cohen *et al.*[7] and Pressman and Sternberger[10] which showed that groups at the active site can be protected from reaction by the prior addition of substrate or competitive inhibitor. The method consists of three basic steps: (1) the treatment of the protein in the presence of substrate with a specific unlabeled reagent until the groups which react with it are saturated; (2) the removal of the substrate; (3) the reaction of the groups at the active site with the same reagent in which a radioactive label has been incorporated.

It is obvious that in order to apply this method of labeling the system under study must fulfill certain requirements. First, the reagent chosen must be capable of

reacting directly with one of the groups at the active site and the covalent bond formed must be stable under the conditions used for protein degradation. Secondly, the substrate-protein complex must be strong enough to protect the active site during the reaction and yet allow the subsequent separation of the substrate without alteration of the protein. Finally, the reaction between the labeled reagent and the active site must be rapid compared to the exchange between the free labeled and the bound unlabeled reagent.

The method has been used successfully to incorporate I^{131} into the active site of antibodies directed against the *p*-azobenzenearsenate grouping. Iodination of antihapten antibody was chosen for these initial studies because previous work[10, 11] indicated that this system possessed most of the necessary properties. It has been shown that an iodine reacting amino acid was present at the active site, that loss of activity during iodination could be prevented through combination with the homologous hapten, *p*-azobenzenearsonic acid, that the various iodine-protein bonds were reasonably stable during either alkaline or enzymatic digestion and, furthermore, that the hapten was easily removable by dialysis. A description of the experimental results and a discussion of the general usefulness of the method follow.

Methods and Material.—Immunizing antigens: The immunizing antigens were prepared by coupling at pH 10, 0°C, 16.6 μM/(2.67g) of purified bovine gamma globulin with the diazonium salt from 2 mM of *p*-aminobenzenearsonic acid. The solutions were then dialyzed in the cold against several changes of 3L batches of 0.15 M NaCl, pH 7.2. Prior to injection the azoglobulin was sterilized by passage through a Seitz filter and then precipitated with alum according to the method of Karush and Marks.[12]

Test antigens: In the test antigens human fibrinogen was substituted for bovine gamma globulin as the protein carrier. A lyophilized preparation of fibrinogen donated by the Red Cross was purified by the method of Laki.[13] Aliquots containing 4 μM (1.36 g) of protein were then coupled to 2 mM of hapten according to the procedure described above. After dialysis in the cold the test antigens were subjected to further purification to remove any adsorbed azo dye. A modification of the procedure of Karush *et al.*[12] was used in which the azofibrinogen was precipitated 5 times from a neutral solution made 3.5 M in NaCl.

Preparation of antisera: The antisera were prepared in rabbits which received multiple injections of the alum-precipitated antigen in increasing dosage over a period of four weeks. Each animal was administered a total of 80 mg of antigen. The animals were exsanguinated on the fourth day after the last injection and the sera which contained more than 100 μg of antihapten antibody N/ml were pooled and frozen.

Purification of antihapten antibody: The initial separation of antihapten antibody from the other serum components was carried out according to the purification scheme of Karush *et al.*[12] The washed immune precipitates were then dissolved in an equal volume of 0.1 M homologous hapten, *p*-aminobenzenearsonic acid, or an analog, *p*-nitrobenzenearsonic acid, adjusted to pH 7.2. Aliquots containing 30 to 40 mg of protein were added to a 1 × 10 cm DEAE column which had been equilibrated with 0.02 M phosphate buffer, pH 7.2. Under these conditions the antibody appeared as a single peak in the eluant and the same peak was observed on rechromatographs of the eluate.

Measurement of antibody: The immunological activity of the purified antibody was assayed by the quantitative precipitin test[14] carried out in duplicate. The *N* content of the specific washed precipitates was measured by the Markham modification[15] of the micro-Kjeldahl method. The results were expressed as the total precipitable antibody/ml as calculated from the Heidelberger and Kendall equation.[16]

Iodination of antibody: Iodine was incorporated into the antibody according to a procedure described by McFarlane[17] in which iodine monochloride equilibrated with carrier-free I^{131} is the iodinating reagent and the reaction is carried out at 0°C in glycine buffer, pH 8.5–9.0. This method had the advantage that the yields were high and that fewer undesirable side reactions occurred per atom of iodine incorporated.

Counting methods: Measurements of radioactivity were made in a deep well scintillation counter. Appropriate corrections were applied for background, decay, and sample volume. To determine specific activities, the antibody samples were precipitated in the presence of 0.13% $NaHSO_3$ and 7% CCl_3COOH; the precipitates were washed three times with 7% TCA, dissolved in dilute NaOH and aliquots removed for counting and N analysis by the micro-Kjeldahl method.

Experimental Results.—Preliminary experiments were undertaken to characterize the reaction between the purified antibody and iodine both in the presence and the absence of hapten.

(a) It was found that the surface reaction groups could be saturated by the successive addition of small amounts of iodine without any appreciable denaturation of the antibody. The data from one of these experiments are summarized in Table 1. Saturation was achieved with the binding of 111 iodine atoms per molecule of

TABLE 1

IODINATION OF ANTIHAPTEN ANTIBODY

Successive Additions of I, Atoms/Molecule	Specific Activity, (c/m/mg) × 10^{-4}	Iodine Bound, Average Atoms/Molecule
44	5.06	40
53	8.74	31
63	11.7	25
65	11.9	2
69	13.4	13
68	12.8	0
	Total	111

antibody and this value was observed to be quite reproducible in other experiments.

(b) When the iodine uptake of pure antibody was correlated with its immunological activity, the results shown in the first 2 columns of Table 2 were obtained. These data confirm the earlier findings using antisera or the gamma globulin fraction of antisera.[18, 19] The binding of less than 5 atoms of iodine per antibody molecule did not significantly alter the ability of the antibody to react with its homologous antigen, while the incorporation of 10 additional iodine atoms destroyed more than 40 per cent of the antibody specificity. It would appear then that the iodination rate of the amino acids at the active site is intermediate in the spectrum of rates exhibited by the surface reactive groups of antibody.

(c) The protective effect of hapten during iodine uptake is illustrated in the last

TABLE 2

PROTECTIVE EFFECT OF HAPTEN DURING IODINE UPTAKE BY ANTIBODY

No Hapten		0.008 *M* Hapten	
Iodine Bound, Average Atoms/Molecule	Loss in Antibody Activity, %	Iodine Bound, Average Atoms/Molecule	Loss in Antibody Activity, %
4.6	0	34	0.6
8.7	19.5	59	43.4
17	44.2	82	54.7
81	100	104	72.0
		107	90.2

2 columns of Table 2. The nitro substituted analog of the specific hapten was used instead of the homologous *p*-aminobenzenearsonic acid because it protected equally well and did not detectably react with iodine. The presence of the hapten provided complete protection for the active site during the incorporation of the first 34 atoms of iodine and gave decreasing protection during the subsequent increments of iodine until near the saturation level less than 10 per cent of the specific activity was recoverable.

(d) The degree of protection was found to vary directly with the hapten concentration. In the presence of 0.001 *M* hapten only 60 per cent of the immunological activity remained after the binding of 30 iodine atoms as compared to essentially 100 per cent in the experiment above. Similarly when the hapten concentration was increased to 0.1 *M*, 89 per cent of the activity was recoverable after the uptake of 77 atoms of iodine as compared to 54.7 per cent with 0.008 *M* hapten. However, even at 0.1 *M* hapten, the highest practical concentration because of the limitations of solubility, saturation of the nonspecific iodine-reacting groups was not achieved without considerable loss of antibody activity.

The conditions used in the labeling of the active site were chosen on the basis of these preliminary experiments. In the initial blocking step the nonspecific reactive amino acids were not totally idoinated because of the observed incomplete protection by hapten at saturation. Amounts of iodine were added which, at the particular hapten concentration employed, gave the maximum blockage of the nonspecific

TABLE 3

LABELING OF THE ACTIVE SITE OF ANTIHAPTEN ANTIBODY

	Exp. 1	Exp. 2
Step 1—Nonspecific Reaction with Cold I		
I added (atoms/mol)	114	47
Estimated I bound (av atoms/mol)	77	40
Hapten conc. (*M*)	0.1	0.005
Step 2—Removal of Hapten		
Dialysis time (hr)	48	24
Over-all loss in antibody activity (%)	11.1	2.0
Step 3—Specific Labeling with I^{131}		
I added (atoms/mol)	4.8	7.2*
I bound (av atoms/mol)	4.6	5.7*
Loss in antibody activity (% of total remaining after step 2)	66.4	63.9*

* Total of 2 iodinations with I^{131}; of each 3.6 atoms added, 3.3 and 2.4 respectively were bound and the losses in antibody activity were 36.5% and 27.4%.

groups without appreciable damage to the active site. It was hoped that under these conditions all the nonspecific groups which reacted more rapidly or equally as rapidly as the amino acids at the active site would be iodinated. Thus, after removal of the hapten, only the active site would be labeled on the addition of a few atoms of radioactive iodine per protein molecule.

The details and the results of two labeling experiments are given in Table 3. It

is immediately apparent that some of the active sites were labeled since the incorporation of as little as 4.6 iodine atoms/antibody molecule destroyed more than 65 per cent of the immunological activity. The data also indicated that some of the radioactivity was not at the active site, since the immunological activity should have been completely destroyed if all the observed protein-bound activity were localized at the active site.

The question of the relative amounts of active site and nonactive site radioactivity was important to resolve because the subsequent isolation of peptides from the active site was dependent on their having a significantly higher specific activity than the peptides from the other regions of the antibody molecule. The minimum amount of bound iodine which would reduce the antibody titer by the observed 65 per cent was calculated from the assumption that the introduction of one iodine atom into either of the two sites on the antibody molecule was sufficient to destroy the precipitating capacity and the known fact[20] that free iodine reacts more rapidly with mono-iodinated than noniodinated tyrosine. The value obtained on this basis was 1.7 iodine atoms/antibody molecule bound to the active site in each experiment. Thus, a maximum of 2.9 radioactive atoms in experiment 1 and 4.0 in experiment 2 were incorporated into nonspecific sites either by exchange with cold protein-bound iodine or by addition to reactive amino acid which had not been previously iodinated.

The exchange between free and protein-bound iodine during iodination was determined from the radioactivity released in the supernatant when I^{131} labeled antibody was treated with cold iodine and then precipitated with TCA. The conditions used in experiment 1 were duplicated: the antibody-hapten complex was treated with 3 successive amounts of radioactive iodine at an iodine-protein ratio of 40 to 1; the hapten was removed and 5 atoms of cold iodine/antibody molecule were added to one-half the solution while an equivalent quantity of glycine buffer was added to the other half. The difference obtained between the control and test supernatants was 3.8 per cent of the total bound radioactivity. From this value the number of radioactive iodine atoms bound by exchange in the labeling experiment 1 was calculated to be 2.9.

It can be concluded, therefore, that exchange essentially accounted for all the radioactivity bound to nonspecific sites. Control experiments showed that the exchange radioactivity was either distributed at random among many sites or was concentrated in a few rapidly exchanging groups from which it would be leeched with unlabeled iodine. In either case, in the final preparation the specific activity of the labeled amino acids at the active site was significantly higher than that of any other iodine reacting amino acid in the molecule.

Discussion.—The experimental results with antihapten antibody have clearly demonstrated the feasibility of the proposed method of labeling. It should be emphasized, however, that the method is not limited to the system studied, but is equally applicable to any protein whose active site can be protected through combination with a specific substrate or competitive inhibitor. Furthermore, the choice of reagent is not limited to iodine, but includes any compound which forms a covalent bond with an amino acid at the active site and which does not inactivate the protein during the initial saturation of groups outside the active site.

In addition to its general usefulness for labeling a single amino acid at the active

site, the method has a feature which is not present in the labeling procedures used for phosphoglucomutase and the esterases. In those cases the determination that serine is at the active site does not establish which, if any, of the amino acids near serine in the sequence are at the active site. Moreover, it is already clear that amino acids distant from each other in the amino acid sequence are present at the active site. Therefore, some way for determining which parts of the protein chain are in contact with the substrate is necessary for the description of the three-dimensional geometry of this area. The method described here can be used for this purpose by successive treatment of a single enzyme with different amino acid reagents. In this way all of the reactive amino acids in contact with the substrate can be identified and the sequences adjacent to them described. Thus, in addition to its use in labeling active sites that cannot be labeled by other procedures, the method will serve to clarify the composition and three-dimensional geometry of active sites in general.

Summary.—A general method has been developed for the labeling of the active site of antibodies and enzymes. It consists of three steps which are (1) the treatment of the protein in the presence of substrate with a specific unlabeled reagent, (2) the removal of the substrate, and (3) the reaction of the groups at the active site with the same reagent in which a radioactive label has been incorporated.

The method has been successfully applied to incorporate I^{131} into the active site of rabbit antibody directed against the *p*-azobenzenearsenate group.

The method is of particular practicality because (*a*) it does not depend on any fortuitous property of the antibody or enzyme and (*b*) it provides a means for mapping the three-dimensional structure of the active site.

* Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[1] Najjar, V. A., and M. E. Pullman, *Science*, **119**, 631 (1954).

[2] Jagannathan, V., and J. M. Luck, *J. Biol. Chem.*, **179**, 561 (1949).

[3] Koshland, D. E., Jr., W. J. Ray, Jr., and M. J. Erwin, *Fed. Proc.*, **17**, 1145 (1958).

[4] Jansen, E. F., M. D. F. Nutting, and A. K., Balls, *J. Biol. Chem.*, **179**, 201 (1949).

[5] Schaffer, N. K., S. C. May, Jr., and W. H. Summerson, *J. Biol. Chem.*, **202**, 67 (1953).

[6] Schaffer, N. K., R. P. Lang, L. Simet, and R. W. Drisko, *J. Biol. Chem.*, **230**, 185 (1958).

[7] Dixon, G. H., R. L. Kauffman, and H. Neurath, *J. Am. Chem. Soc.*, **80**, 1261 (1958).

[8] Cohen, J. A., R. A. Oosterbaan, M. G., Warringa, and H. S. Jansz, *Discussions Faraday Soc.*, No. **20**, 114 (1955).

[9] Hopkins, F. G., E. J. Morgan, and C. Lutwak-Mann, *Biochem. J.*, **32**, 1829 (1938).

[10] Pressman, D., and L. A. Sternberger, *J. Immunol*, **66**, 609 (1951).

[11] Herriott, R. M., *J. Gen. Physiol.*, **20**, 335 (1936–1937); *ibid.*, **25**, 185 (1941–1942).

[12] Karush, F., and R. Marks, *J. Immunol.*, **78**, 296 (1957).

[13] Laki, K., *Arch. Biochem.*, **32**, 317 (1951).

[14] Kabat, E. A., and M. M. Mayer, *Experimental Immunochemistry*, (Springfield: Charles C Thomas, 1948).

[15] Markham, R., *Biochem. J.*, **36**, 790 (1942).

[16] Heidelberger, M., and F. E. Kendall, *J. Exper. Med.*, **61**, 563 (1935).

[17] McFarlane, A. S., *Nature*, **182**, 53 (1958).

[18] Pressman, D., and L. A. Sternberger, *J. Am. Soc.*, **72**, 2226 (1950).

[19] Cohen, S., *J. Immunol.*, **67**, 339 (1951).

[20] Li, C. H., *J. Am. Chem. Soc.*, **64**, 1147 (1942).

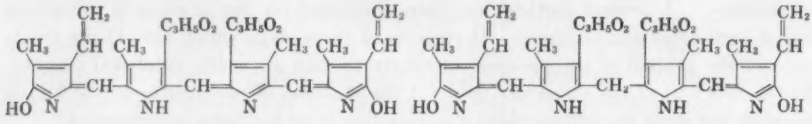
ON THE METABOLIC BREAKDOWN OF HEMOGLOBIN AND THE ELECTRONIC STRUCTURE OF THE BILE PIGMENTS

By Bernard Pullman and Anne-Marie Perault*

INSTITUTE FOR MUSCLE RESEARCH AT THE MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

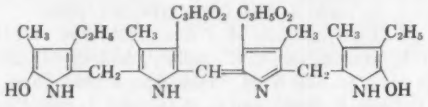
Communicated by Albert Szent-Györgyi, August 24, 1959

The metabolic breakdown of hemoglobin consists, in its first phase, in an oxidative cleavage of the protoporphyrin ring, through the elimination of the methine group situated between the two pyrrole rings carrying the vinyl substituents (α-methine group). The product formed, *choleglobin*, is then easily split into globin, ferric ions and a green pigment, *biliverdin* (I) which is an open chain conjugated totrapyrrole. The further metabolic transformations consist in *a chain of reductions:* biliverdin



(I) Biliverdin (II) Bilirubin

is reduced to bilirubin (II), a pigment of orange color and possibly also to a dark brown pigment, called *urobilin* (III), through the conversion of, respectively, the central or the two terminal methine groups, —CH=, to methylene groups —CH_2—. Further reductions lead through the hydrogenation of all the methine and the vinyl groups to *urobilinogen*, and through the complementary partial hydrogenation of the two terminal pyrrole rings to *stercobilin*.[1]



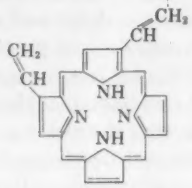
(III) Urobilin

Biliverdin and bilirubin are the principal *bile pigments*.

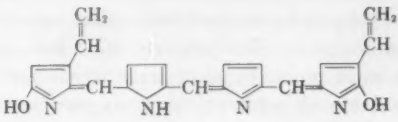
This process of metabolic degradation through *successive hydrogenations* is to some extent peculiar as the general scheme for the metabolic degradation of conjugated biological substances, e.g., purines and pyrimidines of the nucleic acids, consists rather in an extensive series of *successive oxidations*, followed eventually by hydrolysis. The present study was undertaken in view of finding an interpretation for this state of affairs.

The Calculations.—The calculations have been carried out by the molecular orbital method in the L.C.A.O. approximation.[2] They refer to the electronic properties of the π electrons pool of the compounds. The saturated groups present in the molecule have been considered to have only a negligible influence on these properties and have been omitted from calculations. Protoporphyrin has thus been assumed to be simply a 1,4-divinyl porphyrine (IV) and biliverdin has been represented by the simplified formula (V). Bilirubin has been considered to be composed of two separated conjugated fragments VI and VII, which are both substituted

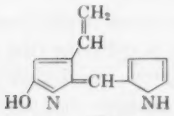
dipyrroles. Dipyrrole VIII represents also the central conjugated fragment of urobilin.



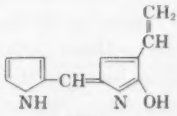
(IV)



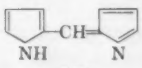
(V)



(VI)



(VII)



(VIII)

A unique value $\alpha_N = \alpha_c + 0.7\beta_{cc}$ has been attributed to the coulomb integral of the four nitrogen atoms of the compounds studied, this value being intermediate between the one that we adopt usually[2, 4] for a pyridine type nitrogen ($\alpha_N = \alpha_c + 0.4\beta_{cc}$) and a pyrrole type nitrogen ($\alpha_{NH} = \alpha_c + \beta_{cc}$). Previous studies, by a series of different authors[5] on properties of the unsubstituted porphyrine ring show effectively that the changes introduced by adopting different values of this parameter for the hydrogen-carrying and hydrogen-free central nitrogens or by considering the different possible tautomeric forms are rather small and irrelevant for the description of the fundamental electronic characteristics of this ring, such as the values of the electronic energy levels or the distribution of the electrical charges.

These two characteristics will, in fact, be those which we shall essentially take into consideration in this paper. May we remind the reader[2, 4] that in the L.C.A.O. molecular orbital method the energies of the molecular orbitals of the mobile or π electrons of the system are expressed in the form $E_i = \alpha + K_i\beta$, where α is the coulomb integral and β the resonance integral of the method. The energies of the individual orbitals are thus characterized essentially by the value of the corresponding K_i. Positive values of K_i correspond generally to orbitals occupied in the ground state of the molecule (bonding orbitals) and negative values of K_i to orbitals empty in that state (antibonding orbitals). The smallest positive value of K_i corresponds to the highest occupied molecular orbital (h.o.m.o) and the smaller this value the greater the electron-donor capacity of the molecule. The smallest negative value of K_i corresponds to the lowest empty molecular orbitals (l.e.m.o) and the smaller this value the greater the electron-acceptor capacity of the molecule.

Results and Discussion.—The table gives the energies of the highest filled and lowest empty molecular orbitals and the figure the distribution of the electrical charges (in electronic units) in the compounds studied.

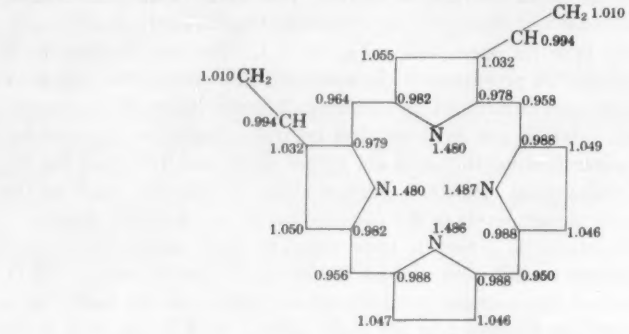
TABLE 1

Compound	Energy of the Highest Occupied Molecular Orbital	Energy of the Lowest Empty Molecular Orbital
Protoporphyrine IV	0,293	−0,233
Biliverdin V	0,455	0,021
Vinyl-hydroxydipyrrole VI	0,468	−0,251
Dipyrrole VIII	0,618	−0,252

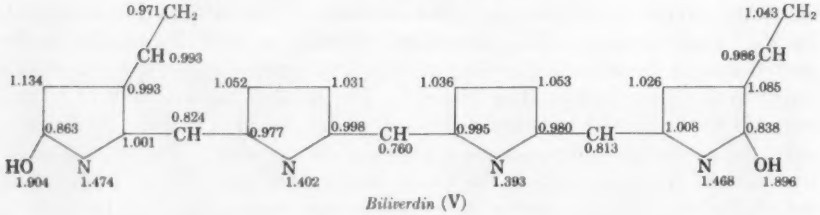
The principal conclusions which may be drawn from these data seem to be the following:

(1) *Protoporphyrin constitutes at the same time a good electron donor and a good electron acceptor.* This property is, in fact, a general characteristic of porphyrins and is, most probably, of primary significance for their biological functioning. In connection with our previous study on the structure and functioning of the coenzymes of oxido-reduction[4] it appears particularly plausible that the electron donor and acceptor properties of the porphyrins are of importance in determining, in conjunction with the similar properties of the central metal atom, the role of the cytochromes as electron carriers.

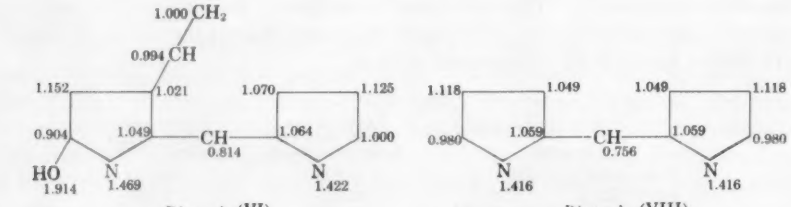
(2) *The oxydative metabolic rupture of the hemoglobin occurs at the electron richest methine bridge.* In fact, all the methine carbons of the porphyrine ring are rather slightly electron deficient, bearing less than the one π electron which they contribute to the electron pool. They carry thus, in fact, a small positive charge (which may be obtained by subtracting from unity their electronic charge). The metabolic rupture of the macro-ring occurs thus at the less positive methine carbon.



Protoporphyrin (IV)



Biliverdin (V)



Dipyrrole (VI) *Dipyrrole* (VIII)

FIG. 1—Distribution of electronic charges.

(3) The rupture of the porphyrine ring and the formation of an opened tetrapyrrole are accompanied by a drastic redistribution of the electronic energy levels and in particular of those corresponding to the highest occupied and lowest empty molecular orbitals. Thus, *biliverdin seems to possess a very unusual property, which has not yet been observed in another compound, namely, that its lowest empty molecular orbital is a bonding one* ($K = 0.021$), the sign of its coefficient being that generally associated with orbitals which are already occupied in the ground state of molecules. This signifies that this molecule must possess unusually pronounced electron acceptor properties. It will be eager to fill up all its bonding orbitals and will consequently be particularly easily reductible. *This characteristic may then be considered as responsible for the apparently uncommon orientation imposed on the mechanism of metabolic transformation of hemoglobin.*†

The possibility thus shown of the existence of compounds possessing the unusual characteristic of a bonding lowest empty orbital is complementary to the possibility which we have recently shown[4-6] of the existence of compounds in which the highest filled molecular orbital is an antibonding one.

(4) The reduction of biliverdin occurs essentially at the central methine carbon and only to a much lesser extent at the terminal ones. In connection with this, it may be remarked that the central methine carbon is particularly greatly electron deficient and will consequently have a strong tendency to complete its electronic gap. Energy considerations also favor the reduction at the central methine bridge: the loss of resonance energy corresponding to this reduction is practically negligible ($0.05\beta \approx 1$ Kcal/mole), while it is of the order of $0.4\beta (\approx 8$ Kcal/mole) for the double reduction on the two terminal methine carbons.‡

(5) Both in bilirubin and in urobilin there still is a conjugated dipyrrole system which will undergo a further reduction. It may be noted that this dipyrrole, whether substituted, as in bilirubin, or unsubstituted, as in urobilin, does not possess any more the unusual property of a bonding lowest empty orbital. Nevertheless, the lowest empty orbital of these dipyrroles, although antibonding, is still a very low-lying one, which means that these systems will still be excellent electron acceptors and, consequently, should be, as they apparently are, easily reductible. (We have calculated the energies only for the vinyl-hydroxy-dipyrrole VI, but there is no doubt that the properties of the isomeric vinyl-hydroxy-dipyrrole VII will be very similar.)

The unusual property of possessing a bonding lowest empty molecular orbital appears to be dependent on the number of pyrrole rings in the polypyrrole chain: it is not present in the dipyrrole chain but manifests itself in the tetrapyrrole chain. (In the unsubstituted tetrapyrrole analog of biliverdin the bonding character of this orbital should be even more pronounced than in biliverdin itself: its $K_t = 0.064$.) We are investigating, at present, the general conditions necessary for the appearance of this unusual property.

This work was sponsored by a grant (C-3073) of the United States Public Health Service. It was prepared while one of the authors (B. P.) was attending the Conference on Submolecular Biology at the Institute for Muscle Research at Woods Hole. This author wishes to thank Dr. Albert Szent-Györgyi for useful discussions.

* Permanent address: Institut de Biologie Physico-Chimique, Université de Paris, 13 rue Pierre Curie, Paris 5e, France.

† It may be reminded that the purines and pyrimidines of nucleic acids, which are metabolically degraded by a series of successive oxidations, are electron donors.[3]

‡ Porphyrins are endowed, in general, with a very great resonance energy which is responsible for the relative stability of these molecules. Thus, the resonance energy of the protoporphyrine IV is 10,570 β, which, with the usual value adopted for β in this type of calculation, namely β = 20 kcal/mole, represents about 210 kcal/mole. Although the rupture of the cyclic macro-ring represents a loss of a part of the resonance energy, this energy is still appreciable in the bile pigments: e.g., the resonance energy of biliverdin is 9,851 β which is nearly 200 kcal/mole.

[1] For more details about these transformations see, e.g., R. Lemberg and J. W. Legge, "Haematin Compounds and Bile Pigments" (New York: Interscience Publishers, 1949), or C. H. Gray, "The Bile Pigments" (London: Methuen Ltd., 1953).

[2] For a general description of the method see, e.g., B. Pullman and A. Pullman. "Le théories électronique de la chimie organique" (Paris: Masson Ed., 1952).

[3] Pullman, B., and A. Pullman, these PROCEEDINGS, **44**, 1197 (1958).

[4] Pullman, B., and A. Pullman, these PROCEEDINGS, **45**, 136 (1959).

[5] E.g., H. C. Longuet-Higgins, C. Rector, and J. R. Platt, *J. Chem. Phys.* **18**, 1174 (1959); T. Nakajima and H. Kon, *J. Chem. Phys.* **20**, 750 (1952); S. L. Matlow, *J. Chem. Phys.* **23**, 673 (1955); G. R. Seely, *J. Chem. Phys.* **27**, 125 (1957); H. Kobayashi, *J. Chem. Phys.* **30**, 1373 (1959).

[6] Pullman, B., and A. Pullman, *Biochim. et Biophys. Acta* (in press). See also G. Karreman, I Isenberg, and A. Szent-Györgyi, *Science* (in press).

A STATISTICAL ANALYSIS OF DEOXYRIBONUCLEIC ACID DISTRIBUTION IN DENSITY GRADIENT CENTRIFUGATION

BY NOBORU SUEOKA

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY

Communicated by Paul Doty, August 25, 1959

Meselson, Stahl, and Vinograd (1957)[1] have established a powerful technique for providing information on molecular weight, density, and their heterogeneities in one operation. A brief description of the way the technique is applied to deoxyribonucleic acid (DNA) is as follows; two to three micrograms of DNA in a 7.7 molal cesium chloride solution of density 1.7 are centrifuged in the SPINCO Model E analytical ultracentrifuge. After an equilibrated density gradient of cesium chloride is established, DNA molecules converge to a position in the gradient corresponding to its density and form a narrow band. Theoretically it has been shown that in the absence of heterogeneities of both density and molecular weight the distribution of DNA molecules is Gaussian with a standard deviation (σ) which is a function of the molecular weight of the DNA sample used. In the presence of molecular weight heterogeneity, both number and weight average molecular weights can be derived from the band profile.[1]

A DNA sample can have, however, both molecular weight and density heterogeneities. Recently it has been shown that the molecular density of DNA is related to its base composition.[2, 3] It has been also shown that heat denaturation[4, 2] and incorporation of either 5-bromouracil,[1] or heavy isotopes of nitrogen[4, 5] and carbon[6] change the density.

In order to analyze the experimental distribution of DNA in the band when both molecular weight and density heterogeneities exist, a general theory is presented and its applications to sonicated molecules of calf thymus and pneumococcus DNA are described.

Scales and Their Transformations.—Because the density gradient is essentially constant within the band range and it has been established that there is a linear relationship between the density and guanine-cytosine content of DNA molecules, three linear scales exist for the discussion of our problem and provide the following linear transformations among them. These are: (1) distance from the center of rotation in centimeter, r, (2) effective density, ρ, and (3) the guanine-cytosine fraction in DNA molecules, p,

$$\rho = \left(\frac{d\rho}{dr}\right)_{\bar{r}} (r - \bar{r}) + \bar{\rho} \quad \text{(reference 1)}$$

$$\rho = 0.103p + 1.662 \quad \text{(reference 2)}$$

$$p = 9.71 \left(\frac{d\rho}{dr}\right)_{\bar{r}} (r - \bar{r}) + \bar{p}$$

where $\bar{r}$, $\bar{\rho}$ and $\bar{p}$ are the means of the distribution in r-, ρ- and p-scales, respectively, and $(d\rho/dr)_{\bar{r}}$ is the density gradient at $\bar{r}$ which can be calculated by equation (38) of reference (7).

The p- and ρ-scales are more convenient than the r-scale, for the statistics on these scales are independent of the conditions applied in particular experiments and are therefore directly comparable. A special advantage of the p-scale is obvious in the case of fragmentation of DNA, which will be discussed later. This scale has also an advantage that the chemically determined guanine-cytosine content can be used as the mean ($\bar{p}$). Among linearly related scales transformations of variance (σ^2) from one scale to another are generally made by means of the relation:

$$\sigma^2(ax + b) = a^2\sigma^2(x)$$

Analysis of Native DNA Distribution.—In general on the x-scale we define the actual distribution of native DNA in a band by a function, $T_N(x)$; similarly the DNA distribution in the absence of thermal agitation (that is, the real density distribution of the molecules) by $D_N(X)$; the distribution caused solely by thermal agitation of the molecules having the value X of $D_N(X)$ by $B_N(X, x)$. Here x and X are on the same scale and X is the mean of $B_N(X, x)$. The subscript N refers to native DNA. Each function is defined as follows:

$$\begin{aligned} \textstyle\int_R T_N(x)dx &= C \\ \textstyle\int_R D_N(X)dX &= C \\ \textstyle\int_R B_N(X, x)dx &= 1 \end{aligned}$$

where R indicates that the integration covers the whole range of the distribution, and C is the total amount of DNA in the band measured by ultraviolet absorption (weight concentration). Now the actual distribution $T_N(x)$ is

$$T_N(x) = \textstyle\int_R D_N(X)B_N(X, x)dX, \qquad (1)$$

and the variance of this function is by definition

$$\sigma_{TN}^2 = \frac{1}{C}\int_R (x - \bar{x})^2 T_N(x)dx,$$

where $\bar{x}$ is the mean of the actual distribution in x-scale. Combining equation (1) and the above we get

$$\sigma_{TN}^2 = \frac{1}{C}\iint_R (x - \bar{x})^2 D_N(X)B_N(X, x)dXdx. \quad (2)$$

As we can put

$$(x - \bar{x})^2 = (x - X)^2 + (X - \bar{x})^2 + 2(x - X)(X - \bar{x}),$$

$$\sigma_{TN}^2 = \frac{1}{C}\iint_R (x - X)^2 D_N(X)B_N(X, x)dXdx + \frac{1}{C}\iint_R (X - \bar{x})^2 D_N(X)B_N(X, x)dXdx + \frac{2}{C}\iint_R (x - X)(X - \bar{x})D_N(X)B_N(X, x)dXdx \quad (3)$$

The first term of equation (3) becomes

$$\frac{1}{C}\int_R D_N(X)\left[\int_R (x - X)^2 B_N(X, x)dx\right]dX = \frac{1}{C}\int_R D_N(X)\,\sigma_{BN_X}^2 dX = \sigma_{BN}^2 \quad (4)$$

where $\sigma_{BN_X}^2$ is the variance of $B_N(X, x)$ and σ_{BN}^2 is the mean variance of B_N functions. The second term similarly becomes

$$\frac{1}{C}\int_R (X - x)^2 D_N(X)\left[\int B_N(X, x)dx\right]dX = \frac{1}{C}\int_R (X - x)^2 D_N(X)dX = \sigma_{DN}^2$$

where σ_{DN}^2 is the variance of $D_N(X)$ or the variance due to density heterogeneity. The third term is zero. Thus,

$$\sigma_{TN}^2 = \sigma_{BN}^2 + \sigma_{DN}^2. \quad (5)$$

Here σ_{BN}^2 should be calculated by equation (9) of reference (1) using a number average molecular weight determined by independent methods.[8] The total variance σ_{TN}^2 is calculated by numerical integration of the observed distribution with Sheppard's correction. Equation (5) shows that the total variance (σ_{TN}^2) calculated from the band profile is the sum of a variance (σ_{BN}^2) caused by Brownian motion of molecules which is molecular weight dependent and a variance (σ_{DN}^2) due to density heterogeneity.

The number average molecular weight (M_N) of native DNA can be obtained from a sedimentation profile in the following way. First the sedimentation constant ($s_{20,w}^{50}$) is obtained from the transition of the 50 per cent points of sedimentation profile along with the time. The weight average molecular weight (M_W) can be obtained from the empirical equation[10]

$$s_{20,w}^{50} = 0.063\, M_W^{0.37}. \quad (6)$$

It should be noted that the value 0.063 is valid only for $s_{20,w}^{50}$. The distribution of sedimentation coefficients ($s_{20,w}$) is obtained by the procedure of Schumaker and Schachman.[9] The ratio, weight average to number average molecular weight (M_W/M_N) can be calculated by

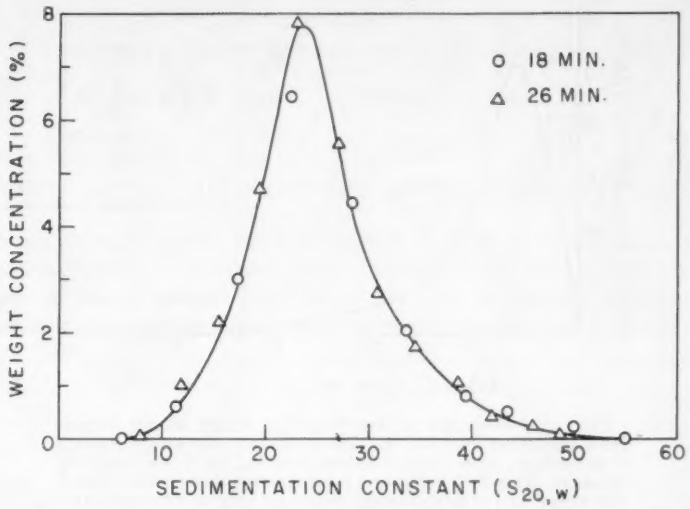


FIG. 1.—Distribution of sedimentation coefficients of native calf thymous DNA calculated from sedimentation profile. The sedimentation pattern was photographed with Kodak X-Ray Film with ultraviolet light. The initial concentration of DNA is 15 μg per ml. The cell depth is 3 cm and the total optical density at 260 mμ at the plateau of the picture is less than 1. Circles were calculated from 18-min picture and triangles from 26-min picture. The tracings of the photographs were made by Joyce double beam microdensitometer and the effective slit width is 0.064 mm. Points for the two different times agree very well indicating the negligible effect of diffusion and convection during the centrifugation.

$$M_W/M_N \simeq \frac{(\sum_i y_i s_i^{2.7})(\sum_i y_i s_i^{-2.7})}{(\sum_i y_i)^2} \qquad (7)$$

(see appendix)

where s_i is a sedimentation coefficient and y_i is the incremental weight fraction of s_i. From the ratio and the M_W, the number average molecular weight can be obtained.

The transformation of the $s_{20,w}$-distribution to the molecular weight distribution is not necessary for the calculation of M_N. It is possible, however, to make such transformation, which may visualize the extent of the molecular weight heterogeneity. The necessary equations are

$$M_i = \frac{M_W s_i^{2.7}}{\beta} \qquad (8)$$

$$Y_i = \frac{0.37 s_i}{M_i} y_i \qquad (9)$$

where

$$\beta = \frac{\sum_i s_i^{2.7} y_i}{\sum_i y_i}. \qquad (10)$$

(see appendix)

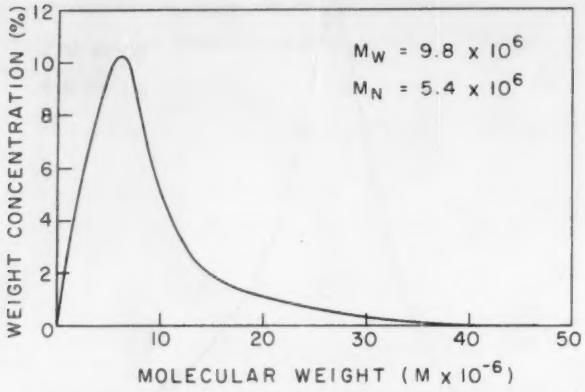


FIG. 2.—Distribution of the molecular weight of calf thymus DNA calculated from Fig. 1. The ordinate is the weight fraction in percentage. The transformation from s_{20w} to M was made by equations (8) and (9) in the text using the smoothed curve of Fig. 1. The weight and number average molecular weights were calculated by the methods described in the text.

The M_i is the molecular weight corresponding to s_i, and Y_i is the weight fraction of M_i. The calculation by equations (7)–(10) are greatly facilitated by making the table of $s^{2.7}$ and $s^{-2.7}$ for discrete values of s from 1 to 100. Figures 1 and 2 show the distribution of $s_{20,w}$ and molecular weight (M) of a native calf thymus DNA. The ratios M_W to M_N calculated for calf thymus and pneumococcus DNA are 1.8 1.2, respectively.

In general the extent of density heterogeneity may be conveniently expressed as percentage by,

$$H_D = \frac{\sigma_T^2 - \sigma_B^2}{\sigma_T^2} \times 100 \qquad (11)$$

where σ_T^2 is the total variance and σ_B^2 is the variance due to thermal agitation. It may be noted that the relative magnitude of the variances in equations (5), (11), and (12) are independent of the scale used.

As examples, analyses on native calf thymus and pneumococcus DNA will be described. Sedimentation coefficient, molecular weights, and ratios (M_W/M_N) are given in Table 1. Tracings of ultraviolet absorption photographs of DNA

TABLE 1

SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF CALF THYMUS AND PNEUMOCOCCUS DNA†

Source of DNA	$s^0_{20,w}$	M_W*	M_W/M_N	M_N*
Calf thymus	24.4	9.8×10^6	1.8	$5.4 \times 10_6$
Pneumococcus	24.9	10.4×10^6	1.2	$8.7 \times 10_6$

* Sodium salt of DNA.
† The calf thymus DNA has been prepared by Simmon's detergent method,[20] and the pneumococcus DNA by a chloroform-octanol method,[21] from *Diplococcus pneumoniae* strain R36A.

TABLE 2

VARIANCES AND DENSITY HETEROGENEITY INDICES (H_D) OF CALF THYMUS AND PNEUMOCOCCUS DNA

Source of DNA	Variances ($\times 10^4$)*			
	$\sigma_{TN}^2(p)$	$\sigma_{BN}^2(p)$	$\sigma_{DN}^2(p)$	H_D, %
Calf thymus	27.8	4.8	23.0	83
Pneumococcus	6.9	3.0	3.9	57

* Variances are given in the p-scale. $\sigma_{BN}^2(p)$ was calculated from $1.33 \times M_N$ which is the number average molecular weight of cesium salt of DNA.

bands are shown in Figure 3 and the calculated variances in the p-scale are summarized in Table 2. The actual (total) variance of calf thymus DNA is four times as large as that of pneumococcus DNA. It is noted that the main part of the difference comes from the large density heterogeneity of the calf thymus DNA.

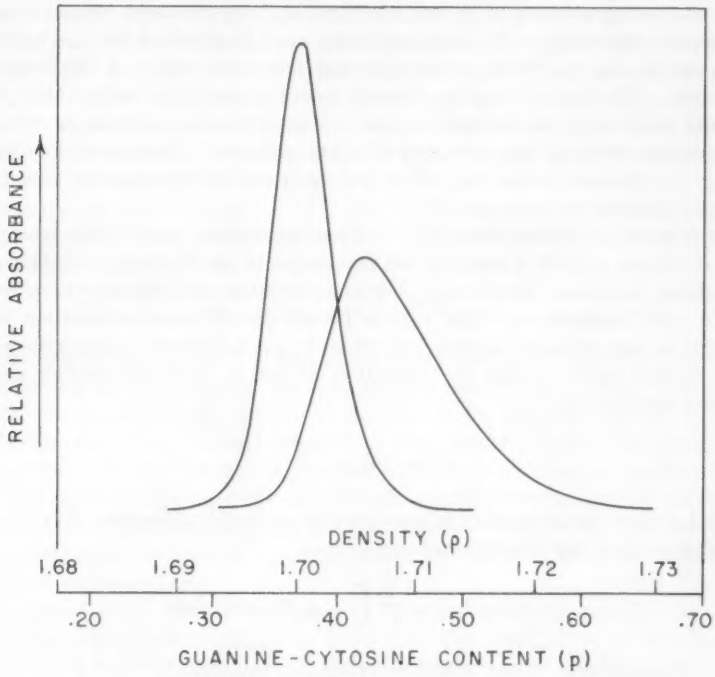


FIG. 3.—Microdensitometer tracings of the ultraviolet absorption photographs of equilibrated calf thymus and pneumococcus DNA in density gradient centrifugation. Both DNA samples were purified by chloroform octanol method, and separately centrifuged at 44,770 rpm for more than 50 hr. Equilibrium was checked by comparing the band profiles at about 12-hr intervals. Tracings were made using a Joyce microdensitometer with an effective slit width (in the cell dimension) of 0.013 mm. The curves have been smoothed to eliminate the background noise normally found in tracings. The area under the curves has been made equal for better visual comparison. The $\bar{p}$ values for calf thymus and pneumococcus DNA adopted in this figure are 0.44 and 0.37, respectively.

Analysis of Fragmented DNA.—In this section we will deal with the analysis of fragmented molecules, where each molecule is split into an average of ν pieces.

General case: We designate the actual distribution of fragmented DNA in the

density gradient field by $T_F(x)$. The intrinsic density distributions of the native and the fragmented DNA in the absence of thermal agitation are given by $D_N(X)$ and $D_F(X, X')$, respectively. Here D_F is the distribution function of density of the fragments originated from the native molecules which had the density X. The thermal distribution of the fragmented molecules having the density X' of $D_F(X, X')$, is expressed by $B_F(X, X', x)$. The variance relationship of these functions is obtained by operations similar to those described before. Thus,

$$\sigma_{TF}^2 = \sigma_{BF}^2 + \sigma_{DN}^2 + \sigma_{DF}^2 \tag{12}$$

or noting $\sigma_B^2 \propto M^{-1}$,

$$\sigma_{TF}^2 = \nu\sigma_{BN}^2 + \sigma_{DN}^2 + \sigma_{DF}^2 \tag{13}$$

Here σ_{TF}^2 is the variance of T_F and σ_{BF}^2 and σ_{DF}^2 are the mean variances of B_F's and D_F's, respectively. The total variance, σ_{TF}^2, is obtained like σ_{TN}^2 from the band profile, and σ_{BF}^2 from number average molecular weight of the fragmented molecules. The value of σ_{DN}^2 is obtained from the analysis of native DNA. The residual variance is an estimate of σ_{DF}^2. This estimate, however, is subject to accumulated errors of the estimates of other variances. Independent estimation of σ_{DF}^2 is, therefore, desirable, which will be discussed for sonicated and heated samples in the following sections.

Application for sonicated molecules: Upon sonication, each DNA molecule is cut into pieces without separating the two chains of the Watson-Crick helix.[10] If we assume a random distribution of guanine-cytosine and adenine-thymine pairs within each molecule and that σ_{DN}^2 is totally due to base composition heterogeneity, we can calculate an expected value of σ_{DF}^2 as follows: taking the p-scale, the variance newly arising by sonication of native molecules having guanine-cytosine content p is

$$\sigma'_{DF_p}{}^2(p) = \frac{p(1-p)}{b},$$

where b is the average number of base pairs in sonicated molecules. For the entire distribution, however, the expected variance is

$$\begin{aligned}\sigma'_{DF}{}^2(p) &= \frac{1}{C}\int_R \sigma'_{DF_p}{}^2(p)D_N(p)dp \\ &= \frac{1}{Cb}\int_R p(1-p)D_N(p)dp \\ &= \frac{1}{b}\left[\bar{p}(1-\bar{p}) - \sigma_{DN}^2(p)\right]\end{aligned} \tag{14}$$

where $\bar{p}$ is the average guanine-cytosine content of the DNA sample. Here upon sonication it is assumed that $\bar{p}$ remains as the average of the sonicated sample.[22] This provides a possibility to test the randomness of the pair distribution within the molecule, by comparing σ_{TN}^2 with the sum of the three variances (σ_{BF}^2, σ_{DN}^2 and $\sigma'_{DF}{}^2$). If the distribution of two kinds of base pairs within the molecule is predominantly nonrandom, the sum is expected to be larger than σ_{TN}^2, while smaller

TABLE 3

MOLECULAR WEIGHTS AND VARIANCES OF SONICATED CALF THYMUS AND PNEUMOCOCCUS DNA

Source of DNA	$s^{0}_{20,w}$	M_W	Variances ($\times 10^4$)* $\sigma_{TF}^2(p)$	$\sigma_{BF}^2(p)$	$\sigma_{DN}^2(p)$	$\sigma'_{DF}^2(p)$	H_D, %
Calf thymus	9.7	0.83×10^6	79.8	61.5	23.0	2.6	23
					87.1		
Pneumococcus	12.5	1.6×10^6	37.4	31.9	3.9	0.8	15
					36.6		

* Variances are given in the p-scale. $\sigma_{BF}^2(p)$ was calculated from the half of $1.33 \times M_W$ assuming M_N is approximately half of M_W. The factor 1.33 is for converting sodium salt to cesium salt of DNA. The $\bar{p}$ values for calf thymus and pneumococcus DNA used in calculation of $\sigma'_{DF}^2(p)$ are 0.44 and 0.37, respectively.

if a regular nonrandoness (e.g., a tendency toward alternation) is dominant. The analysis of sonicated samples of calf thymus and pneumococcus DNA[11] is summarized in Table 3. The ratios, M_W/M_N, calculated directly from sedimentation pattern are 2.5 for calf thymus 2.8 for pneumococcus. These values are, however, too large for small molecules because of the effect of diffusion. Since electron micrographs of sonicated calf thymus DNA give the ratio approximately two[12] in the present analysis the ratio two is used.

Application for heated molecules: We can apply equation (13) as such for heat denatured molecules. If we assume that two component chains of the double helix separate by heating, the variance equation (13) becomes,

$$\sigma_{TF}^2 = 2\sigma_{BN}^2 + \sigma_{DN}^2 + \sigma_{DF}^2 \qquad (15)$$

Effective density of a heated DNA increases by a universal value, 0.015,[2, 4] so that the linear relationship between guanine-cytosine content and density maintains its slope, allowing to use the same scale for σ_{DF}^2 as for the other terms. A theoretical estimation of σ_{DF}^2 should be obtained from random deviation of guanine or cytosine from $p/2$ and adenine or thymine from $(1 - p)/2$ among single chains. Unfortunately we do not have experimental data on the effective density difference between guanine and cytosine or adenine and thymine. The value, σ_{DF}^2, however, will be quite small because of the large number of nucleotides in the denatured molecule of the native DNA.

Discussion.—It has been shown[13] that if the macromolecular material examined by the density gradient centrifugation has a Gaussian distribution of the effective density, it will form a Gaussian band and thus appears to be homogeneous. The variance of the resulting distribution in this case is a simple sum of the two component variances, one for the effective density distribution and the other for thermal agitation. Consequently it has been pointed out that with the presence of density heterogeneity, the molecular weight estimated by this technique gives an underestimation. However, the two assumptions, homogeneous molecular weight and Gaussian distribution of effective density, are not satisfied in general. Furthermore, it has been shown that DNA from calf thymus, salmon sperm and mouse have skewed distributions.[7, 2] The solution given in this paper is general and can be applied for the cases in which both density and molecular weight heterogeneities are present; it has no restrictions on the mode of their distributions.

It is apparent from earlier work[2, 13] that the principal cause of density hetero-

TABLE 4

RANGE OF BASE COMPOSITION DISTRIBUTION IN GUANINE-CYSTOSINE FRACTION OF CALF THYMUS AND PNEUMOCOCCUS DNA ESTIMATED FROM THREE DIFFERENT METHODS

Source of DNA	Density* Gradient Centrifugation $[\bar{p} \pm 2\sigma_{DN}(p)]$	Hyperchromic† Effect by Heating $(\bar{p} \pm 2\sigma)$	Chemical‡ Fractionation
Calf thymus	0.34–0.54	0.33–0.55	0.40–0.48[14] 0.40–0.55[15] 0.34–0.52[16]
Pneumococcus	0.33–0.41	0.33–0.41	...

* The $\bar{p}$ values for calf thymus and pneumococcus adopted in this table are 0.44 and 0.37, respectively.
† See reference (18).
‡ Ranges were taken from the upper and lower extreme values of the references indicated.

geneity in DNA is the compositional variation among the molecules of a given sample. The adequacy of this explanation can be checked to some extent by comparing the estimates of composition heterogeneity obtained by two other methods (fractionation and absorbance-temperature curves) with that obtained from the analysis of density gradient band profiles. The existence of such heterogeneity in base composition has been analyzed chemically by fractionation of native molecules of DNA of calf thymus,[14–16] pig liver,[14] sea urchins,[14] *E. coli*,[16] and T_{2r}[16] and T_6[14] phages. Another evidence came from hyperchromic effect of DNA by thermal inactivation.[17, 18] The range of the heterogeneity detected by these three methods in calf thymus and pneumococcus DNA is summarized in Table 4. These data agree fairly well and therefore we can conclude that at least most of the density heterogeneity is due to base composition variation among the molecules of calf thymus and pneumococcus DNA.

The molecular weight which is used to calculate the σ_B^2 should be the number average molecular weight. The method of obtaining the number average molecular weight described in this paper is not exact but the approximation involved does not appear to be serious. This approximation is based on the fact that the exponent α of equation (1′) of the appendix is constant while the coefficient, K, is subject to small variation depending on the molecular weight distribution within the sample and on the deviation of the mean and the median of the sedimentation distribution. Experimental data at present do not appear to justify a more elaborate approach to the number average molecular weight. In addition the procedure used here is simple and generally applicable.

If there is no density and molecular weight heterogeneity present, the sonicated DNA (Table 3) should have more than nine times larger variance in calf thumus DNA and five times larger variance in pneumococcus DNA than those of the native molecules. The actual variance of the sonicate, however, is found to be only three times as large as the native variance in calf thymus DNA which indicates the presence of a large proportion of density heterogeneity. A fair agreement between the total variance and sum of three variances (σ_{BF}^2, σ_{DN}^2, σ'^2_{DF}) in both calf thymus and pneumococcus DNA indicates that the distribution of adenine-thymine and guanine-cytosine pairs within molecule is not far from random in these cases.

Our analysis of variances has some relevance to the conclusions that have been reached concerning the halving of the molecular weight by heating in *E. coli* DNA,[8] while in calf thymus and salmon sperm DNA no change in band width was ob-

served. From equation (15) it is clear that even if the molecule does become half, the band width will not increase very much in case of the large relative values of σ_{DN}^2 which remains in the equation. Therefore, it is quite possible that calf thymus and salmon sperm DNA reduce the molecular weight to half by heating but that the reduction is essentially undetectable from a comparison of the band width.

Appendix.—When sedimentation coefficient, s, and molecular weight, M, have the relation,

$$s = KM^{\alpha} \tag{1'}$$

and the weight fractions for s and M are y and Y, respectively, the following relations are obtained.

$$yds = YdM \tag{2'}$$

$$M_W = \frac{\int_R YM dM}{\int_R Y dM} = \frac{\int_R s^{1/\alpha}\, yds}{K^{1/\alpha}\int_R y ds} \simeq \frac{\sum_i s_i^{1/\alpha} y_i}{K^{1/\alpha}\sum_i y_i} \tag{3'}$$

$$M_N = \frac{\int_R Y dM}{\int_R \frac{Y}{M}\, dM} = \frac{\int_R y ds}{K^{1/\alpha}\int_R s^{-1/\alpha} y ds} \simeq \frac{\sum_i y_i}{K^{1/\alpha}\sum_i s_i^{-1/\alpha} y_i} \tag{4'}$$

The R indicates the integration covers the whole range of the distributions. Therefore,

$$M_W/M_N = \frac{(\int_R s^{1/\alpha} yds)(\int_R s^{-1/\alpha}\, yds)}{(\int_R y ds)^2} \simeq \frac{(\sum_i s_i^{1/\alpha} y_i)(\sum_i s_i^{-1/\alpha} y_i)}{(\sum y_i)^2} \tag{5'}$$

Combining (1') and (3') and putting

$$\beta = \frac{\int_R s^{1/\alpha} yds}{\int_R yds} \simeq \frac{\sum_i s_i^{1/\alpha} y_i}{\sum_i y_i} \tag{6'}$$

we get,

$$M_i = \frac{M_W s_i^{1/\alpha}}{\beta} \tag{7'}$$

From (1') we get,

$$\frac{ds}{dM} = \alpha K M^{\alpha-1} = \alpha \frac{s}{M}$$

Combining this with (2′) and (6′) we get,

$$Y_i = \frac{\alpha s_i}{M_i} y_i \qquad (8')$$

For $\alpha = 0.37$ we obtain the equations (7), (8), (9), and (10) from (5′), (7′), (8′), and (6′), respectively. A transformation from $s_{20,w}$-distribution to M-distribution has been discussed by Williams and Saunders for integral form of distribution.[19]

The author is deeply indebted to Professor P. Doty for his constant encouragement and valuable suggestions. The pneumococcus DNA and its sedimentation picture were kindly supplied by Dr. J. Marmur, the sonicated calf thymus and the sonicated pneumococcus DNA were given by Mr. J. Eigner. Criticisms and suggestions by Dr. E. Freese and Dr. R. D. Luce are greatly acknowledged. The author is also indebted to Mr. J. Kucera for technical help. This work was supported by research grant E-1421 from the United States Public Health Service to Prof. R. P. Levine, from a National Science Foundation Grant, G7025 to Profs. R. P. Levine and J. D. Watson, and from a United States Public Health Service Grant C-2170 to Prof. P. Doty.

[1] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43,** 581 (1957).

[2] Sueoka, N., J. Marmur, and P. Doty, *Nature*, **183,** 1429 (1959).

[3] Rolfe, R., and M. Meselson, these PROCEEDINGS, **45,** 1039 (1959).

[4] Meselson, M., and F. W. Stahl, these PROCEEDINGS, **44,** 672 (1958).

[5] Sueoka, N., "Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi*" (in press).

[6] Davern, C., *J. Molecular Biology* (in press).

[7] Meselson, M., *Ph.D. Thesis*, California Institute of Technology (1958).

[8] It is clear from equation (4) of the present paper and equation (18) of reference (1) that σ_{BN}^2 is inversely proportional to the number average molecular weight. The same DNA preparation should be used for the density gradient centrifugation and sedimentation coefficient determination.

[9] Schumaker, V. N., and H. K. Schachman, *Bioch. Bioph. Acta*, **23,** 628 (1957).

[10] Doty, P., B. B. McGill and S. A. Rice, these PROCEEDINGS, **44,** 432 (1958).

[11] For the conditions of sonication see M. Litt, J. Marmur, H. Ephrussi-Taylor and P. Doty, these PROCEEDINGS **44,** 144, 1958 and P. Doty, B. B. McGill, and S. A. Rice, these PROCEEDINGS, **44,** 432 (1958).

[12] Hall, C., and P. Doty, *J. Am. Chem. Soc.*, **80,** 1269 (1958).

[13] Baldwin, R. L., these PROCEEDINGS, **45,** 939 (1959).

[14] Crampton, C. F., R. Lipshitz and E. Chargaff, *J. Biol. Chem.*, **211,** 125 (1954).

[15] Bendich, A., H. B. Pahl, G. C. Korngold, H. S. Rosenkranz and J. R. Fresco, *J. Am. Chem. Soc.*, **80,** 3949 (1958).

[16] Brown, G. L., and A. V. Brown, *Symposia of the Society for Experimental Biology*, **12,** 6 (1958).

[17] Marmur, J., and P. Doty, *Nature*, **183,** 1427 (1959).

[18] Doty, P., J. Marmur and N. Sueoka, *Proceedings of Brookhaven Symposium* (in press).

[19] Williams, J. W., and W. Saunders, *J. Phys. Chem.*, **58,** 854 (1954).

[20] See Rice, S. A., and P. Doty, *J. Am. Chem. Soc.*, **79,** 3937 (1957).

[21] Marmur, J., in preparation.

[22] *Note added in proof:* It was found recently that sonication does not affect the density of either calf thymus or pneumococcus DNA.

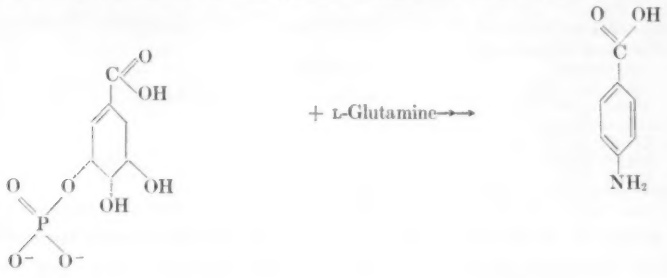
*THE BIOSYNTHESIS OF p-AMINOBENZOIC ACID**

By B. Weiss and P. R. Srinivasan

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY

Communicated by D. Rittenberg, August 3, 1959

Nutritional studies with mutants of *Escherichia coli*[1] and *Neurospora*[2] have established shikimic acid as an intermediate in the biosynthesis of phenylalanine, tyrosine, tryptophan, and *p*-aminobenzoic acid. Subsequent investigations have revealed other mutants which accumulate shikimic acid-5-phosphate (I) or "Z1" in their medium and require all the above compounds for growth.[3] Compound "Z1" (probably the 5-enolpyruvate of shikimic acid[4]) originates at a later stage in the biosynthetic sequence by a condensation of shikimic-5-P and phosphoenolpyruvate.[5] These observations suggest that "Z1" may be the branching point in the formation of these aromatic compounds in nature. However, recent work with enzyme extracts of *E. coli* mutants have shown that anthranilic acid (the benzenoid intermediate in the biosynthesis of tryptophan) is formed from shikimic-5-P and L-glutamine.[6] This finding has led us to study the biosynthesis of *p*-aminobenzoic acid as well. We demonstrate in the present paper that *p*-aminobenzoic acid is formed in cell-free extracts of yeast from shikimic-5-P and L-glutamine.



(I)

Material and Methods.—Shikimic acid-5-phosphate was isolated from the culture medium of *Aerobacter aerogenes* strain A 170–40 as the barium salt[7] and converted to the potassium salt for the enzymatic experiments. Yeast concentrate and liver concentrate were obtained from Sigma Chemical Co., St. Louis.

Preparation of enzyme extract: 50 g of baker's yeast were disrupted by freezing and thawing three times with the aid of liquid nitrogen. The viscous material was stirred with 50 ml of *M*/10 potassium phosphate buffer (pH 7.4) for 4 hours at 2°, centrifuged at 15,000 g for 20 minutes and the cell-free extract was extensively dialyzed against *M*/30 potassium phosphate buffer (pH 7.4; 6 changes, 3 liters each time). The dialyzed extract was used in the experiments described below.

Enzymatic system and assay of p-aminobenzoic acid: The incubation mixtures contained 0.4 ml of extract (4.8 mg of protein), 2 μmoles of $MgCl_2$, 1 mg of yeast concentrate, 50 μmoles of Tris buffer (pH 7.4), shikimic-5-P or glutamine, plus additions in a total volume of 1 ml. After incubation at 30°, samples were removed at 4, 8, and 24 hours, brought to pH 1.0 with 6 *N* HCl, and used for the microbiological determination of PABA with a double auxotroph of *E. coli* (107–14) requir-

ing histidine and PABA.[8] The assay medium (final volume 10 ml) was prepared by supplementing mineral medium A[9] with 75 μg of L-histidine per ml.

Conversion of uniformly labeled C^{14}-shikimic-5-P to p-aminobenzoic acid:[10] 312 μmoles of C^{14}-shikimic-5-P, 1560 μmoles of L-glutamine, 625 mg of yeast concentrate, 3125 μmoles of tris buffer (pH 7.4), 1250 μmoles of $MgCl_2$ and 250 ml enzyme extract in a total volume of 650 ml, were incubated for 24 hours at 30°. After incubation, the mixture was placed in a boiling water bath for 10 min, cooled, and the pH adjusted to 3.0 with 4 ml of 6 N H_2SO_4. The precipitated protein was removed by centrifugation, and aliquots of the supernatant solution were analyzed for PABA at three levels in duplicate. To 625 ml of this supernatant solution, which contained 2.29 mg of PABA, 100 mg of unlabeled PABA were added. The PABA was then isolated by continuous ether extraction and was recrystallized from H_2O to constant radioactivity and melting point.

Results and Discussion.—Amino and carbon donors: Of the various amino donors tried with shikimic-5-P, glutamine was the most effective (Table 1). Aspartic acid, asparagine, and glutamic acid were poorly utilized. Ammonium chloride was unable to replace glutamine as the amino donor at either pH 7.4 or 8.2.

TABLE 1

SYNTHESIS OF *p*-AMINOBENZOIC ACID FROM SHIKIMIC-5-P AND AMINO DONORS*

Substrates and Additions	Yield of *p*-Aminobenzoic Acid (μg) 4 Hours	8 Hours	24 Hours
0.5 μmole of shikimic-5-P	0	0	0
+ L-glutamine	2.4	4.1	4.70
+ L-glutamic acid	...	...	0.06
+ L-asparagine	...	...	0.09
+ L-aspartic acid	...	...	0.11
+ NH_4Cl (pH 7.4)	...	...	0.05
+ NH_4Cl (pH 8.2)	...	...	0.05

* 2.5 μmoles of each amino donor were used in these experiments.

With glutamine as the amino donor, various carbon sources were studied for their capacity to substitute for shikimic-5-P (Table 2). Shikimic acid alone was poorly

TABLE 2

SYNTHESIS OF *p*-AMINOBENZOIC ACID FROM L-GLUTAMINE AND VARIOUS CARBON SOURCES

Substrates and Additions	Yield of *p*-Aminobenzoic Acid (μg) 4 Hours	8 Hours	24 Hours
2.5 μmoles of glutamine	0.88	0.80	0.88
+ 0.1 μmole of shikimic-5-P	1.90	2.50	2.60
+ 0.5 μmole of shikimic-5-P	2.40	3.70	4.80
+ 0.5 μmole of shikimic acid	1.30	1.40	1.50
+ 0.5 μmole of shikimic acid + 0.5 μmole of ATP	2.50	2.50	3.00
+ 0.5 μmole of Z1	0.09	0.07	0.06

utilized and addition of ATP enhanced the synthesis to half the value obtained using shikimic-5-P as the carbon source. "Z1" was not only inactive but reduced the blank value considerably, suggesting an inhibitory effect on the synthesis. Irrespective of the amino donor employed, *p*-hydroxybenzoic acid was not converted to PABA. This confirms the earlier observations of Professor B. D. Davis derived from a study of the growth requirements of *E. coli* aromatic auxotrophs.[11] These results demonstrate that shikimic-5-P is the carbon donor in the formation of PABA. The increased synthesis obtained by the addition of ATP to shikimic acid indicates

the presence of a kinase in these extracts for the phosphorylation of shikimic acid to shikimic-5-P.

Glutamine alone added to the extracts without shikimic-5-P resulted in a significant synthesis, indicating the presence of possible precursors in the yeast extract itself. The extracts were therefore assayed for shikimic acid and shikimic-5-P.[12] A typical extract, with a synthesis of 0.9 μg of PABA from glutamine alone, was found to contain 11 μg of shikimic acid and 22.5 μg of shikimic-5-P per ml. The presence of these amounts of substrate could satisfactorily explain the formation of PABA from glutamine in the absence of any extraneous addition of shikimic-5-P. It was further observed that extracts with low blank values could not be stored in the deep freeze even for a few days without losing considerable activity suggesting that the enzyme or enzymes responsible for the conversion of shikimic-5-P and L-glutamine to PABA are protected by their own substrate. The apparent presence of such large amounts of substrate even after extensive dialysis is of interest. It simulates the well-known tight binding of coenzymes in other enzyme systems.

Since the amount of PABA formed was small, it was felt desirable to establish, unequivocally, the nature of the end product. With this view in mind, uniformly labeled C^{14}-shikimic-5-P and L-glutamine were incubated with enzyme extracts, under conditions described earlier in detail. The molar specific activity of the isolated *p*-aminobenzoic acid compared favorably with the molar specific activity of shikimic-5-P, suggesting a 1:1 conversion (Table 3). This experiment lends

TABLE 3

CONVERSION OF SHIKIMIC ACID-5-PHOSPHATE-C^{14} TO *p*-AMINOBENZOIC ACID

Compound	Activity of Carbon* cpm
Shikimic acid-5-phosphate (barium salt)	127×10^3
p-Aminobenzoic acid†	133×10^3

* Counts per minute at "infinite" thickness under standard conditions divided by the fraction of carbon in the compound.
† Corrected for dilution.

further evidence to the identity of the enzymatic product as PABA.

Nature of co-factors: The dialyzed yeast extracts were completely inactive unless they were fortified with yeast concentrate or liver concentrate (Table 4). The

TABLE 4

CO-FACTOR REQUIREMENTS FOR THE SYNTHESIS OF *p*-AMINOBENZOIC ACID

Additions	Yield of *p*-Aminobenzoic Acid (μg) 4 Hours	8 Hours	24 Hours
None	0.11	0.10	0.21
Yeast concentrate (1 mg)	2.80	3.20	4.00
Liver concentrate (1 mg)	2.50	2.60	2.70
Boiled extract of *E. coli* B_{37}*	2.20	2.60	3.00
Yeast concentrate (1 mg) + DPNase	1.36	1.34	1.50

The incubation mixture contained 0.5 μmole of shikimic-5-P; 2.5 μmoles of L-glutamine; 2 μmoles of $MgCl_2$; 50 μmoles of Tris buffer, pH 7.4; 0.4 ml of enzyme extract plus additions in a total volume of 1 ml.
* Kindly furnished by Professor J. S. Gots.

yeast and liver concentrates employed in these experiments contain a mixture of nucleoside monophosphates, diphosphates, DPN, TPN, CoA, and possibly trace amounts of nucleoside triphosphates. *Neurospora* DPNase[13] decreased the synthesis of PABA, in the presence of yeast concentrate, by 50–60 per cent. How-

ever, neither DPN nor DPN + ATP were able to replace the yeast concentrate. Thus, the exact nature of the co-factors involved is obscure, and studies are in progress toward its elucidation.

Since anthranilic acid and *p*-aminobenzoic acid are both derived from shikimic-5-P and L-glutamine, it would be of interest to determine whether a common intermediate may not be involved in the biosynthesis of these two compounds.

Summary.—With cell-free extracts of baker's yeast, it has been demonstrated that *p*-aminobenzoic acid is formed from shikimic acid-5-phosphate and L-glutamine. The co-factor requirements of the system are also briefly discussed.

We are extremely grateful to Professor B. D. Davis for providing us with the mutants used in this work.

* This investigation was supported by a research grant from the National Institutes of Health, United States Public Health Service.

The following abbreviations have been used: shikimic-5-P, shikimic acid-5-phosphate; PABA, *p*-aminobenzoic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosinetriphosphate; CoA, Coenzyme A; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

[1] Davis, B. D., *J. Biol. Chem.*, **191**, 315 (1951); *Advances in Enzymology*, **16**, 287 (1955).

[2] Tatum, E. L., in *Plant Growth Substances*, ed. F. S. Koog (Madison: University of Wisconsin Press, 1951).

[3] Davis, B. D., and E. S. Mingioli, *J. Bact.*, **66**, 129 (1953).

[4] Gilvarg, C., and B. D. Davis, unpublished observations; Davis, B. D., *Arch. Biochem. Biophys.*, **78**, 497 (1958).

[5] Kalan, E. B., and B. D. Davis, unpublished observations.

[6] Srinivasan, P. R., *J. Am. Chem. Soc.*, **81**, 1772 (1959).

[7] Weiss, U., and E. S. Mingioli, *J. Am. Chem. Soc.*, **78**, 2894 (1956).

[8] We are indebted to Dr. E. Borek for providing us with this mutant, which was originally isolated by Dr. B. D. Davis.

[9] Davis, B. D., and E. S. Mingioli, *J. Bact.*, **60**, 17 (1950).

[10] C^{14}-shikimic-5-P was isolated from the culture medium of *Aerobacter aerogenes* strain A 170-40 grown in the presence of uniformly labeled glucose-C^{14}.

[11] Davis, B. D., *J. Bact.*, **64**, 729 (1952).

[12] Shikimic acid was estimated microbiologically (Davis, B. D. and U. Weiss, *Arch., Exp. Path. U. Pharmakol.*, **220**, 1, 1953) and shikimic-5-P was determined by assaying the skimic acid released after cleavage with acid phosphatase.

[13] *Neurospora* DPNase was a generous gift of Professor N. O. Kaplan.

---

*GLUCOSE INHIBITION AND THE DIAUXIE PHENOMENON**

BY ELLIS ENGLESBERG

DEPARTMENT OF BIOLOGICAL SCIENCES, UNIVERSITY OF PITTSBURGH

Communicated by M. Demerec, August 14, 1959

Salmonella typhimurium, strain LT2, is able to utilize glucose, glycerol, pyruvate, and Krebs cycle compounds such as citrate, succinate, fumarate, and malate as sole sources of carbon and energy for growth. Spontaneous mutants (C^-) have been isolated from this strain which have retained the wild type ability to utilize the Krebs cycle compounds but are unable to utilize glucose or other carbohydrates, glycerol, or pyruvate as sole carbon and energy sources.[1] Although growth factor

amounts of aspartate and glutamate, as well as citrate, will bring about excellent growth of these mutants with glycerol as sole carbon and energy source, there is negligible growth when glucose is substituted for glycerol. In fact, the addition of glucose to a medium containing citrate as sole carbon and energy source severely inhibits growth and selection occurs for glucose resistant mutants. It was during a study of the nature of glucose sensitivity and resistance that it became apparent that *Salmonella typhimurium* and the glucose resistant mutants might be of value in the elucidation of the mechanism of the diauxie phenomenon.[2]

Materials and Methods.—*Salmonella typhimurium* LT2[3] employed in this study was obtained from M. Demerec. Wild type and mutant stocks were maintained on nutrient agar (Difco) slants. For various experiments cells were grown in nutrient broth (Difco) or nutrient agar (Difco) or in a synthetic medium of the following per cent composition: KH_2PO_4, 0.3; K_2HPO_4, 0.7; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.01; distilled water and carbon sources as will be indicated.[4] In the preparation of solid media, 1.5 per cent agar was employed. In some experiments the mineral agar medium was enriched with dehydrated nutrient broth (Difco) to yield a 0.01 per cent solution. The carbon source and salts were autoclaved separately and added as concentrated sterile solutions.

Quantitative growth experiments were conducted employing test tubes (growth tubes) 18×150 mm, containing a total of 5 ml of culture medium. The tubes were incubated at 37°C at a 5° angle from the horizontal on a rotary shaker operating at 115 rpm, with an eccentric of 9 mm in radius. At various time intervals, tubes were removed from the incubator and growth determined in a Fisher Electrophotometer modified to take the growth tubes. Growth is recorded in Fisher units (O.D.×100) and is based upon the average of duplicate tubes. Inocula grown under various conditions, washed or unwashed, were employed in the different experiments and will be described in the appropriate place. With an exponentially growing culture of LT2 in a 0.05 per cent glycerol, 0.05 per cent malate mineral medium, 1 unit on the Fisher equals 1.5×10^7 viable cells.

Phage PLT 22[3] stocks were maintained in T2 buffer[5] and stored in the refrigerator. These stocks were prepared by infecting an aerated log phase culture of *Salmonella typhimurium*, containing about 2×10^8 cells/ml, growing in nutrient broth at 37°C with PLT 22 at a multiplicity of 0.1–0.2. Lysis of the culture usually occurred after 3–4 hr additional incubation. The lysates were spun at low speed (3000–5000 rpm) for 20 min to get rid of the bacterial debris and the phage was spun down subsequently at 10,000 rpm (Servall SS-2) for 1 hr. The phage pellet was resuspended in T2 buffer and sterilized by chloroform treatment. Phage assays were made by the standard agar-layer technique.[6]

Transduction experiments were performed by infecting with PLT 22 at a multiplicity of 10 a log phase culture grown at 37°C with aeration (2×10^8 cells/ml). The infected culture was maintained at 37°C for 15 min and plated in triplicate on the appropriate selective medium, and colonies were counted after 48 hr incubation at 37°C.

Acid phosphatase activity was determined by measuring the liberation of inorganic phosphate[7] from glucose-6-phosphate and other organic phosphate esters. Optimum conditions for acid phosphatase activity of extracts, determined with glucose-6-phosphate as substrate, are as follows: sodium maleate buffer pH 5.0,

150 μM; Na glucose-6-phosphate, 20 μM; cell extract; in a total volume of 2.0 ml; final pH, 5.1. When other substrates were tested, similar conditions of pH and substrate concentrations were employed. The reaction mixture was prepared in small test tubes in an ice bath. 0.5 ml samples were removed at zero time, i.e., just after the addition of extract, to 0.5 ml of 10 per cent cold trichloroacetic acid. The reaction mixture was then incubated at 30°C and at various intervals additional samples were removed and treated in a similar fashion. The trichloroacetic acid inactivated samples were maintained in an ice bath for 10 min, 1 ml of distilled water was added, the precipitate was removed by centrifugation, the supernatant fluid was collected, and aliquots removed for inorganic phosphate determinations.[7] Activity recorded is based upon the amount of inorganic phosphate liberated during the first 10-min period after addition of extract, and is expressed in terms of the μM of inorganic phosphate liberated per hour per mg of protein.[8]

Adenosine triphosphatase (ATPase) activity was determined by measuring the liberation of inorganic phosphate[7] from adenosine triphosphate (ATP) in essentially the same manner as described above and is recorded in similar units. The reaction mixture and near optimum conditions for the determination of the activity of this enzyme are as follows: sodium maleate buffer, 150 μM, pH 6.0; sodium adenosine triphosphate (pH 6.0), 40 μM; $MgCl_2$, 20 μM; cell extract, 0.1 ml (approximately 2.0 mg of extract protein); in a total volume of 2.0 ml.

Pyrophosphatase was determined by measuring the liberation of inorganic phosphate[7] from sodium pyrophosphate (see above). The reaction mixture and near optimum conditions for the determination of the activity of this enzyme are as follows: sodium pyrophosphate, 100 μM; tris (hydroxymethyl) aminomethane buffer, 150 μM, pH 9.0; $MgCl_2$, 20 μM; cell extract, 0.10 ml; in a total volume of 2.0 ml.

Glucokinase activity was measured manometrically.[9] The reaction mixture consisted of the following: $KHCO_3$, 81 μM; sodium iodoacetate, 6 μM; KF, 200 μM; $MgCl_2$, 20 μM; sodium adenosine triphosphate, 20 μM; glucose, 20 μM; 0.5 ml of cell extract; total volume, 2.1 ml; atmosphere, 5 per cent CO_2, 95 per cent N_2. A control vessel containing the above ingredients less glucose was employed as a measure of ATPase activity. The reaction was carried out at 30°C. The rate of glucokinase activity, expressed as the number of μM of glucose esterified per hour per mg of protein,[8] is based upon the amount of CO_2 liberated during the second 5 min period after addition of ATP, less the amount of CO_2 liberated by ATPase alone.

6-Phosphoglucose isomerase activity was determined by measuring the production of fructose-6-phosphate (determined as fructose[10] or keto sugar[11]) from glucose-6-phosphate. The reaction mixture and near optimum conditions for the determination of the activity of this enzyme are as follows: tris (hydroxymethyl) aminomethane buffer, 75 μM, pH 7.0; sodium glucose-6-phosphate, 10 μM; cell extract, 0.05 ml; total volume, 1.0 ml. The rate of enzyme activity is expressed as the number of μM of fructose produced per hour per mg of protein, and is based upon the amount of fructose formed during the first 10 min after addition of enzyme extract. Activity was measured at 30°C.

Isocitric dehydrogenase[12] was determined by measuring triphosphopyridine nucleotide (TPN) reduction spectrophotometrically at 340 mμ in a Beckman DU

spectrophotometer. The reaction mixture contained tris (hydroxymethyl) aminomethane buffer, pH 7.0, 30 μM; $MnCl_2$, 1 μM; TPN, 125 μgm; dl-sodium isocitrate, 8 μM; 0.1 ml of a $^1/_{20}$ dilution of cell extract (0.14–0.15 mg of protein); total volume, 3.0 ml. The blank contained all of the above except substrate. Isocitric dehydrogenase activity is based upon the amount of TPN reduced during the second 30 sec period after the addition of substrate, and is expressed in terms of the μM of isocitrate oxidized per hour per mg of protein.

Enzyme extracts were prepared from cells grown in mineral glucose medium which was distributed in 250 ml amounts per liter Erlenmeyer. Each flask was inoculated with 20 ml of an overnight nutrient broth culture and incubated at 37°C on a shaker which rotated at 250 rpm with an eccentric of 1.3 cm in radius. The cells were harvested by centrifugation in the cold when growth had reached a turbidity of 400 (6–7.5 hr of incubation) as measured in the Klett-Summerson Colorimeter using a sterile medium blank with a 425 filter. The supernatant was decanted, the insides of the centrifuge bottles were washed with cold distilled water and the cells were resuspended in distilled water and respun. The washed cells were resuspended in cold distilled water to yield a 15 per cent solution on a wet weight basis and treated in the Raytheon Sonic Oscillator (9KC) for 30 min in the cold. A clear opalescent solution was collected after clarification of the sonicated material by centrifugation (Spinco, 30 head, 10,000 rpm, 30 min).

Results.—The isolation and characterization of the C^- mutants: Salmonella typhimurium, strain LT2, will grow in a complete synthetic medium containing glucose as sole carbon and energy source. By means of the penicillin technique, employing carbon starved LT2, three independent C^- spontaneous mutants (C^{-1}, C^{-2}, C^{-3}) were isolated. These mutants were originally characterized as being able to grow on a yeast extract casein hydrolysate mineral glucose agar medium but not on mineral glucose agar. Further analysis of the mutants by streaking on mineral agar plates containing various carbon sources (in the presence or absence of nutrient broth enrichment) indicated that besides glucose these mutants were unable to utilize any other carbohydrate, glycerol, or pyruvate as sole sources of carbon and energy, while still retaining the wild type ability to grow on citrate, succinate, malate, and fumarate. Further analysis showed that growth on glycerol occurred with the addition of growth factor amounts (50 gamma/ml) of *l*-glutamate and *l*-aspartate, or 100 gamma/ml of citrate, while with glucose as carbon source little or no stimulation was noted by the addition of these compounds. Furthermore, although the addition of glycerol in varying amounts (0.01–2.0 mg/ml) to 0.2 per cent citrate mineral agar plates yielded similar growth or stimulated growth as compared to growth on plates containing citrate alone, the addition of glucose to citrate mineral agar strikingly inhibited growth. Employing C^{-1} it was found that with an inoculum which gave 100–200 colonies on the surface of a mineral citrate agar plate, the addition of glucose to the extent of 2 gamma/ml completely inhibited the appearance of these colonies. The symbol dg^s shall be used to designate this sensitivity to glucose. A further explanation of this choice of symbol will be given later. The glucose nonutilizing, glucose sensitive mutants shall therefore be designated C^-dg^s. Further work to be reported will be confined to the C^{-1} mutant (henceforth to be referred to simply as C^-) and its derivatives.

Isolation of glucose resistant mutants: When C^- is streaked heavily on to a

0.2 per cent citrate plus 0.2 per cent glucose mineral agar plate, secondary mutant colonies appear in 48 hr and continue to arise with further incubation. These mutant clones were isolated by restreaking several times on mineral glucose-citrate agar and slanted. Tests of several of these mutants on agar media demonstrated that they were still unable to grow with glucose, glycerol, or pyruvate as sole carbon and energy sources, but were insensitive to the glucose inhibition, i.e., growth occurred on citrate-glucose medium. One such resistant mutant, studied further, derived from C^-dg^s, shall be referred to as C^-dg^{r-1}.

Isolation of glucose utilizing, glucose resistant variants: To elucidate the nature of the glucose sensitivity and resistance in *Salmonella typhimurium*, an attempt was made to isolate variants which would be isogenic with the wild type for all markers

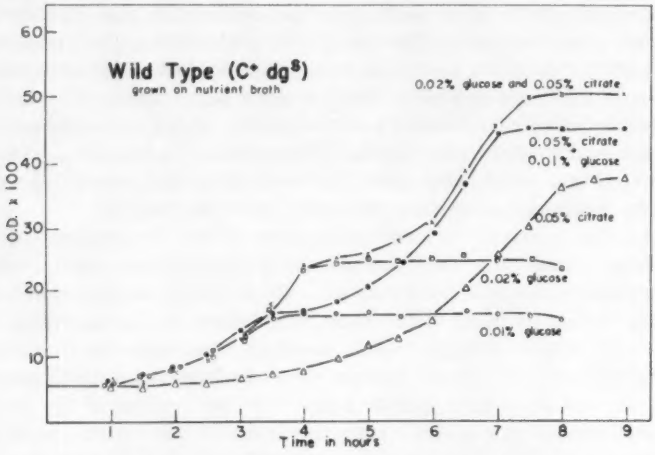


FIG. 1.—The glucose-citrate diauxie phenomenon exhibited by *Salmonella typhimurium*, strain LT2 (wild type), C^+dg^s. Inoculum: 0.2 ml of a nutrient broth culture at a turbidity of 50 (O.D. × 100) as determined in test tubes 150 × 18 mm (650 filter; blank, sterile nutrient broth).

except the glucose resistance marker, so that they might be compared with the wild type physiologically. From C^-dg^{r-1}, C^+ clones were isolated in two ways: Phage PLT 22, previously grown on the wild type and treated with UV to facilitate the isolation of nonlysogenic cells[13] was employed in introducing the C^+ marker back into C^-dg^{r-1}. C^+ transduced clones were selected for on mineral glucose enriched agar plates.[14] One such C^+ transductant was employed in all further analysis. The alternative method of isolation was selection of spontaneously occurring C^+ mutants of C^-dg^{r-1} on mineral glucose enriched medium. The isolation of spontaneous reversions to C^+ was found difficult to accomplish at first, due to the occurrence of what appear to be "suppressor" mutations (C^{+su}) which occur at approximately 10 times the rate of true reversions. These "suppressor" mutants were found to require the nutrient broth enrichment for utilization of glucose as carbon and energy source. However, apparently true reversions of the C^- locus were finally isolated, and these behaved identically to the C^+ isolated by transduction.

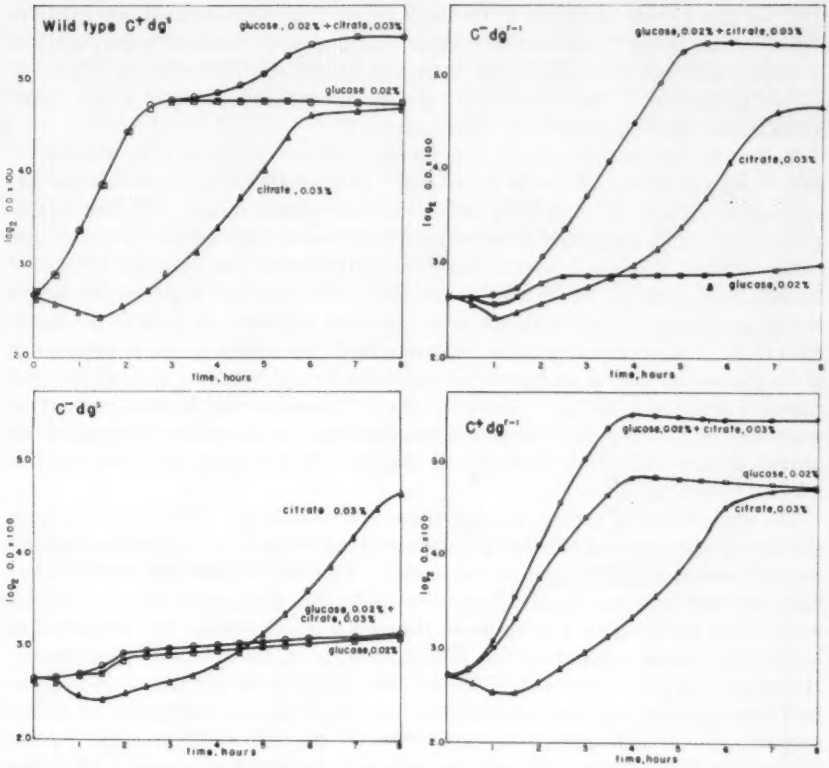


FIG. 2.—Comparison of the growth of *Salmonella typhimurium*, LT2 (wild type) C^+dg^+ and variants C^-dg^+, C^-dg^{r-1}, and C^+dg^{r-1} (produced by transduction of C^-dg^{r-1}). Inoculum: Cells were grown in a mineral 0.05 per cent glycerol, 0.05 per cent malate medium to a turbidity of 50 (O.D. × 100) (see Fig. 1), spun down, washed in saline, and resuspended in saline to a turbidity of 87 (425 filter; blank, sterile mineral medium). 0.2 ml of this suspension was employed as an inoculum.

Growth studies on agar plates of the purified C^+ clones derived from C^-dg^{r-1} and maintained on nutrient agar slants demonstrated that these variants grow on mineral glucose agar and on mineral citrate-glucose agar.

Quantitative growth studies with the wild type and the various glucose sensitive and resistant variants: A quantitative study of the growth characteristics of the wild type and the three related variants was undertaken, and some representative results are shown in Figures 1 and 2. Figure 1 shows the typical growth response of the wild type previously grown in nutrient broth. Note that growth in a mixture of 0.01 per cent glucose and 0.05 per cent citrate results in a typical diauxie type of growth curve. Growth initially follows that given in a medium with 0.01 per cent glucose alone. A break in the curve occurs at the same O.D. at which growth stops, with cells growing with just 0.01 per cent glucose as carbon source, and exponential growth resumes after a lag period. A similar correspondence is found between growth on 0.02 per cent glucose alone and growth on 0.02 per cent glucose +

0.05 per cent citrate, as shown in the same figure. As demonstrated previously in *Aerobacter aerogenes*[15], these results suggest that glucose is used first to the exclusion of citrate, and that when the supply of glucose is exhausted, the cells are induced to citrate utilization. The exclusion by glucose of the utilization of other carbohydrates has also been previously demonstrated.[2]

A clear distinction between all four strains is shown in Figure 2, in which glycerol + malate grown cells were employed. This synthetic medium has the advantage of supporting excellent growth of all four strains tested. All four strains go through a long lag period before exponential growth with citrate as carbon and energy source. Glucose is used as a carbon source by the wild type and by the C^+ transductant, but not by either C^- variant. The addition of glucose to citrate results in diauxic growth with the wild type and inhibition of growth on citrate with C^-dg^s. Mutation to glucose resistance by C^-dg^s results in the neutralization of the glucose inhibition, and growth occurs with the utilization of both glucose and citrate without any diauxie. Similarly, the C^+ transductant, besides growing on glucose alone, still possesses the glucose resistance marker, as indicated by utilization of both glucose and citrate without any diauxie. This mutant, therefore, shall be referred to as C^+dg^{r-1}.

The explanation of the glucose inhibition is now evident. With the wild type, the diauxie phenomenon between glucose and citrate indicates that glucose excludes the utilization of citrate as a carbon source. Glucose is used first, then citrate. Since the wild type can apparently synthesize its own glutamate and aspartate by way of the Krebs cycle from glucose through internal citrate, the inhibition of exogeneous citrate utilization for this purpose is of no apparent consequence. However, with the C^- mutant, which cannot synthesize its glutamate and aspartate from glucose and may be considered an aspartate and glutamate or citrate requirer, glucose still inhibits the utilization of external citrate required for the synthesis of these amino acids and this results in inhibition of growth. Mutation to glucose resistance leads to the growth of C^- in the presence of glucose. The incorporation of this glucose resistance marker (dg^{r-1}) into the wild type genome leads to the abrogation of the glucose diauxie phenomenon and offers proof that glucose inhibition is a direct result of the glucose diauxie phenomenon, a relationship which has been previously implied in Aerobacter.[15] Thus mutation to glucose resistance is mutation to diauxie resistance.

It is apparent from these experiments that the glucose inhibition of citrate utilization cannot be an inhibition of citrate metabolism in the Krebs cycle, otherwise the wild type itself would not be able to utilize glucose as sole carbon and energy source. It will be seen in Figure 2 that *Salmonella typhimurium* and its variants go through a long lag period before exponential growth on citrate. Warburg experiments have shown that cells grown in the absence of citrate (glycerol plus malate grown cells or fumarate grown cells) are unable to oxidize citrate, while subsequent to a short period of growth in the presence of citrate given as sole carbon and energy source, the cells are now able to do so. Cell-free extracts of both types of cells, however, oxidize citrate at similar rates and have high and similar isocitric dehydrogenase and aconitase activities. *Salmonella typhimurium* is, therefore, similar to *Pseudomonas fluorescens*[16] and *Aerobacter aerogenes*[15] with respect to citrate utilization. With these two organisms, the induction period

required for utilization of citrate by the intact cells is associated with the induced synthesis of a protein concerned with the permeation (penetration and/or concentration and/or transport) of citrate to the site of enzyme activity; a factor probably similar to the β-galactosidase permease.[17] Results indicate, therefore, that the glucose inhibition of citrate utilization is at the level of the citrate permeation factor.

Glucose-citrate diauxie phenomenon with citrate grown C^-dg^s: Although nutrient broth or glycerol + malate grown C^-dg^s are almost completely inhibited from growth on citrate by glucose, citrate induced C^-dg^s are not so inhibited, and growth occurs in a citrate + glucose medium. This growth is characterized by a short diauxie lag phase (Fig. 3), which occurs after approximately 2 divisions, and is

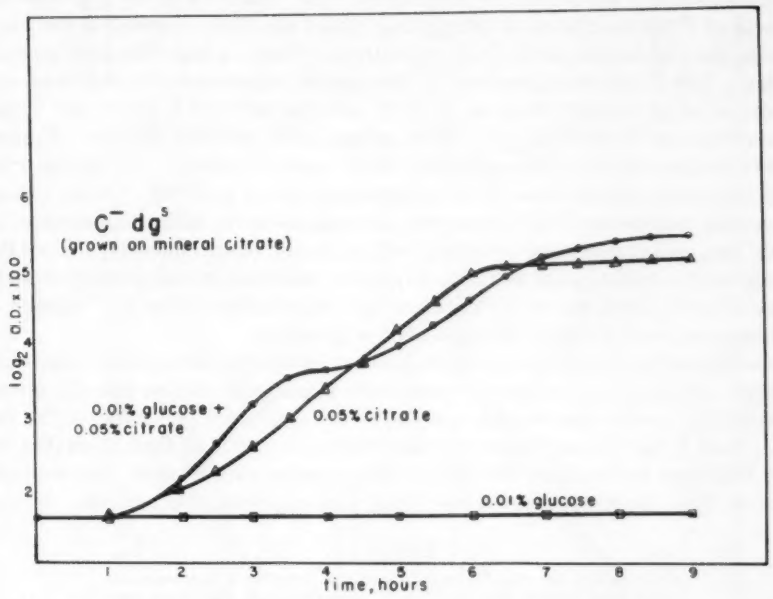


FIG. 3.—Growth of C^-dg^s in a glucose plus citrate mineral medium subsequent to induction to citrate utilization. Inoculum: 0.2 ml of a 0.05 per cent citrate mineral medium at a turbidity of 50 (see Fig. 2).

initiated probably by the exhaustion of the glucose in the medium. Since the amount of growth at this first break (Fisher units 10.4) is what would be expected on the basis of growth of the prototroph on glucose alone, it is apparent that citrate is being utilized mainly, if not exclusively, as a source of glutamate and aspartate during this first phase of growth. The slope of the diauxie lag is 0.2 divisions per hour, which probably represents growth on citrate alone, and is approximately what one might expect on the basis of the glucose initially present inhibiting synthesis of the citrate permeation system, resulting in the diluting out of this system by cell division. Thus when glucose utilization is completed, the cells must go through an induction period before they regain their full capacity for growth with citrate as

sole carbon and energy source. The evidence presented, therefore, indicates that glucose does not inhibit directly the penetration of citrate into the cell and its utilization by C^-dg^s for glutamate and aspartate synthesis, but rather it inhibits the synthesis of the citrate permeation factor. Similar results were obtained with the wild type.

Attempts at transduction of the dg^{r-1} marker: Attempts to transduce the dg^{r-1} marker by growing phage on either C^-dg^{r-1} or C^+dg^{r-1} and infecting C^-dg^s and selecting for glucose resistant clones on mineral glucose + citrate enriched agar have been unsuccessful. High titer phage suspensions as achieved with wild type bacteria are obtained with phage grown on either of the above strains, and these phage are able to transduce C^+ and various auxotrophic markers, and yet are unable to transduce dg^{r-1} at detectable frequencies. The inhibitory effect of glucose on growth of C^-dg^s on glucose + citrate agar plates cannot be responsible for these results, since transductants to C^+dg^s (prototroph) occur at a high frequency on such plates. This is further emphasized by the negative results also obtained in experiments in which several divisions of C^-dg^s infected with PLT 22 (C^+dg^{r-1}) are allowed to occur in nutrient broth before plating on the selecting medium. Experiments furthermore have demonstrated that in crosses in which C^-dg^s was infected with increasing concentrations of phage previously grown on C^+dg^{r-1}, there was no detectable increase in C^-dg^{r-1} over the spontaneous level, while transduction to C^+dg^s occurred at increasing frequency with increasing phage employed. Since the background of spontaneous mutation to glucose resistance averaged approximately 1 per 10^7 cells plated, the possibility exists that transduction of the dg^{r-1} marker, if it does occur at all, occurs at an unusually low frequency.

Specificity of the mutation to glucose resistance: Wild type *Salmonella typhimurium* exhibits a diauxie type of growth curve with mixtures of glucose plus rhamnose, glucose plus l-serine, glucose plus maltose, as well as glucose plus citrate. On the other hand, C^+dg^{r-1} is immune to the diauxie phenomenon in all these cases (Fig. 4). The wild type also exhibits the diauxie phenomenon with fructose plus maltose, fructose plus citrate, galactose plus citrate, and galactose plus maltose. In this

TABLE 1

COMPARISON OF THE GROWTH RATES OF *Salmonella typhimurium* LT2 (WILD TYPE) C^+dg^s AND C^+dg^{r-1} WITH GLUCOSE, FRUCTOSE, AND MANNOSE AS SOLE CARBON SOURCES

Strains	Growth Rate*		
	Glucose	Fructose	Mannose
C^+dg^s	1.13	1.00	0.80
C^+dg^{r-1}	0.77	1.00	0.80

* Growth rate: divisions/hour during exponential growth. Inoculum: 0.2 ml of a washed suspension at a turbidity of 87 (see Fig. 2) of an exponentially growing culture in mineral glycerol plus malate medium. Medium:Mineral medium (see *Materials and Methods*) containing 0.02% carbohydrate.

case, C^+dg^{r-1} behaves exactly like the wild type. Thus mutation to diauxie resistance, as exemplified by this particular glucose resistance marker (dg^{r-1}), leads to diauxie resistance specifically where glucose, and only glucose, is a member of the diauxie pair.

Explanation of symbols: On the basis of these findings, we have chosen the symbols dg^r and dg^s to designate diauxie resistance and diauxie sensitivity, respectively, with regard to glucose. Thus the wild type is C^+dg^s, being able to utilize glucose

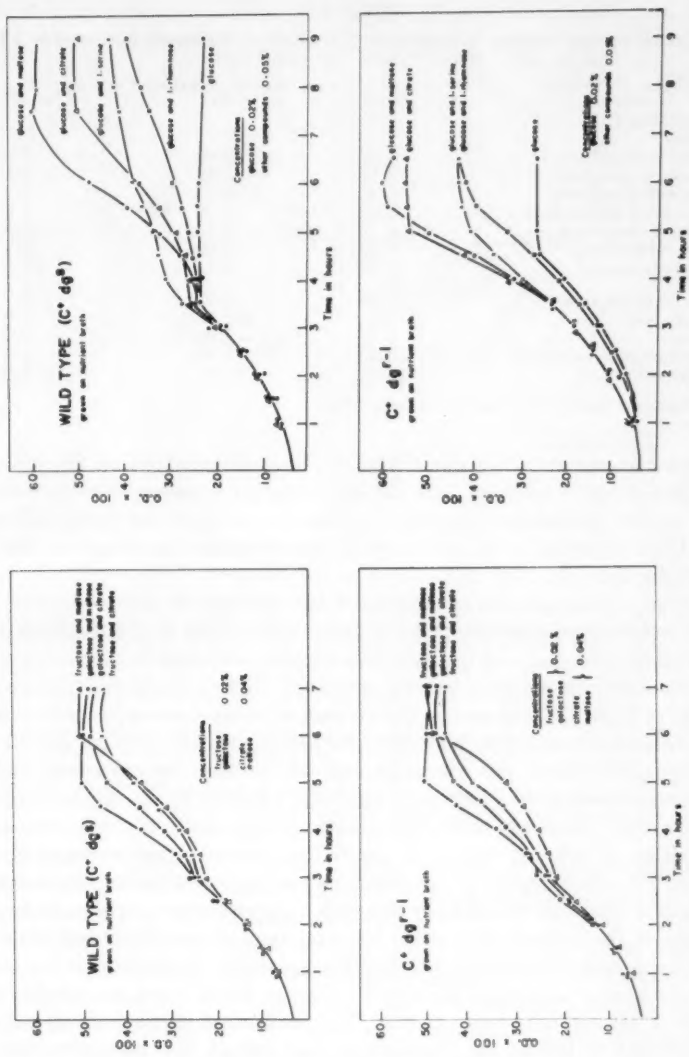


FIG. 4.—The specificity of the dg^{r-l} marker. Inoculum: 0.2 ml of a nutrient broth culture at a turbidity of 50 (see Fig. 1).

and other carbohydrates as sole carbon and energy source, and being sensitive to the glucose inhibition, which, in the case of this organism, is phenotypically expressed as a glucose diauxie. A summary of the mode of origin of the variants employed in these experiments and others mentioned is seen in Figure 5.

The dg^{r-l} marker as a modifier controlling the rate of growth on glucose. A comparison of the growth rate of the wild type and $C^{+}dg^{r-l}$ on glucose, fructose, and mannose is shown in Table 1. It will be noted that the dg^{r-l} marker effects a decrease in growth rate specifically with glucose as substrate.

TABLE 2

COMPARISON OF THE ENZYME ACTIVITIES OF EXTRACTS OF *Salmonella typhimurium* LT2 (WILD TYPE) C^+dg^s AND C^+dg^{r-1}

Enzyme	Activity of Extracts* C^+dg^s	C^+dg^{r-1}	C^+dg^s/C^+dg^{r-1}
Acid phosphatase (pH 5.1)			
Substrates			
Glucose-6-phosphate	3.97	10.90	2.8
Mannose-6-phosphate	2.57	5.45	2.1
Fructose-6-phosphate	2.45	5.28	2.2
Fructose-1,6-diphosphate	3.20	9.29	2.9
Glucose-1-phosphate	4.18	8.10	1.9
Ribose-5-phosphate	2.72	5.90	2.2
Phenyl-phosphate	1.93	3.44	1.8
Adenosine triphosphatase	4.35	4.92	1.1
Pyrophosphatase	141.0	147.0	1.0
Glucokinase	2.17	2.03	0.9
Glucose-6-phosphate isomerase	3.19	3.63	1.1
Isocitric dehydrogenase	46.0	51.0	1.1

* See *Materials and Methods* for explanation of activity units.

Enzymatic analysis of C^+dg^s and C^+dg^{r-1}: Enzymatic analysis of the wild type (C^+dg^s) and C^+dg^{r-1} revealed that the dg^{r-1} marker causes a two- to threefold increase in acid phosphatase activity.[18] There is no other significant difference that could be detected in the activities of other enzymes in extracts of the two strains (Table 2).

Discussion.—Although the microbial cell has the genetic potentiality for producing a multitude of enzymes, many of these enzymes are not synthesized unless the cell comes in contact and reacts with a specific compound in the environment, usually the substrate for the particular enzyme. Recent evidence indicates that formation of inducible enzymes in the absence of inducer are repressed by intracellular components, and that the inducer releases the enzyme forming system from this repression.[19] Thus the microbial cell has evolved an extremely efficient mechanism preventing the synthesis of enzymes, which at any particular time may not be required. Similarly microorganisms have apparently evolved mechanisms for utilization of external stimuli as controlling factors in repression of enzyme formation. A culture placed in a medium containing two utilizable carbon sources, one or both of which are attacked by inducible enzyme systems, may in many cases utilize one of the compounds first to the exclusion of the other, and when this primary compound is completely utilized then induction of enzymes for the utilization of the second compound occurs.[2,15] Glucose is the most powerful of such inhibitors of induced enzyme synthesis. The mechanism of the utilization of such external stimuli to inhibit the synthesis of what are, at that particular time and under most circumstances, useless enzymes have been commented upon and studied by many workers,[2, 15, 20–23] and will not be reviewed in any detail in this paper.

In this investigation it has been shown that the glucose repression of induced enzyme formation, which in the wild type, in essence, is an efficient control mechanism, results in death in a deficient mutant like C^-. By a series of mutation and transduction steps, it has been possible to construct an organism which is isogenic to the wild type except for the incorporation of a glucose resistance marker (dg^{r-1}). By means of this variant, it has been shown that glucose sensitivity and resistance are a direct ramification of the glucose diauxie effect, and that immunity to this

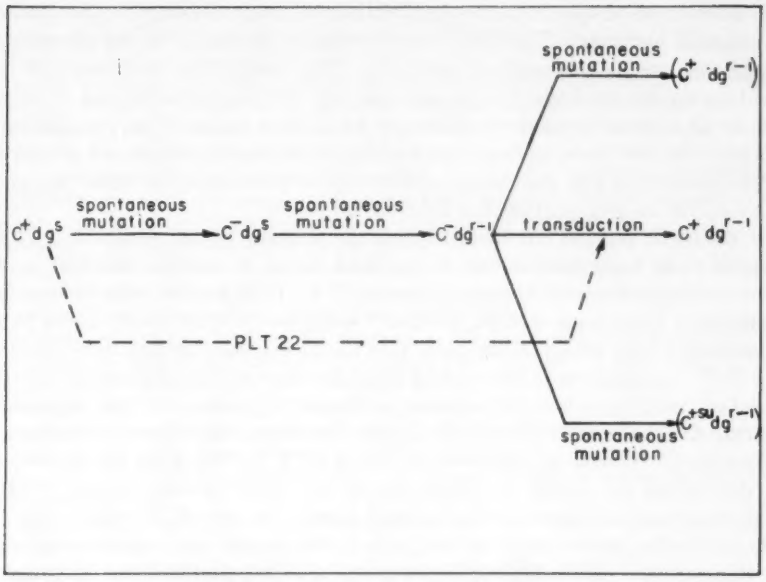


FIG. 5.—Derivation of mutants. C^+dg^s: Wild type, able to use glucose, glycerol, or pyruvate as sole carbon and energy source; shows the glucose diauxie phenomenon. C^-dg^s: Carbohydrate, glycerol, and pyruvate non-utilizing mutant isolated from the wild type by the penicillin technique; glucose diauxie phenotypically expressed by glucose inhibition of growth on citrate. C^-dg^{r-1}: Carbohydrate, etc., nonutilizing, glucose resistant mutant isolated as a spontaneous mutant from C^-dg^s on glucose plus citrate agar plates; grows on glucose plus citrate without diauxie. C^+dg^{r-1}: Isogenic with the wild type except for the dg^{r-1} marker; uses carbohydrates, etc., as sole carbon and energy source; immune to the glucose diauxie; isolated by transduction. (C^+dg^{r-1}): As above; isolated as a spontaneous mutant on mineral glucose enriched medium. $(C^{+su}dg^{r-1})$: Grows on carbohydrates, etc., only in the presence of nutrient broth enrichment; immune to the glucose diauxie; isolated by selection of a spontaneous mutant on mineral glucose enriched plates.

effect, as typified by the dg^{r-1} marker, is specific for glucose diauxie alone, and leads to a decreased growth rate, specifically with glucose as substrate, and a two- to threefold increase in acid phosphatase activity.

Besides the potential significance of these findings with regard to the elucidation of the mechanism of the glucose diauxie, the interaction of the dg^{r-1} marker on the prototrophic phenotype presents an ideal model on an enzyme level as to the functioning of modifier genes.[24] The increased phosphatase activity caused by the dg^{r-1} marker can be considered to modify the functioning of the genes involved directly in glucose metabolism, by partially short circuiting glucose utilization at the phosphorylated stage, resulting in the lowering of the growth rate with glucose.

The correlation between decreased growth rate, increased acid phosphatase activity, and diauxie resistance and the deficiency of C^-dg^s suggests two hypotheses to explain the glucose diauxie phenomenon. (1) The phosphorylated and nonphosphorylated intermediates in glucose metabolism, up to, but not including, Krebs cycle compounds and those produced from Krebs cycle compounds are the repressors of induced enzyme formation in this system.[1] (2) Inorganic phosphate is limiting during glucose metabolism.[25] The inorganic phosphate may be required

for the synthesis of specific factors (e.g., ribonucleic acid) necessary for the synthesis of inducible enzymes. The liberation of inorganic phosphate by the phosphatase would then enable this synthesis to occur. This competition for phosphate may occur during the synthesis of enzymes concerned with glucose metabolism (competition on an enzyme forming level) and/or as a direct result of the metabolism of glucose. On this basis, one would expect that a decrease in the amount of enzymes directly concerned with glucose metabolism and/or liberation of inorganic phosphate as a result of the phosphatase may have similar results.

It should be pointed out that although the glucose enzyme system is generally referred to as being constitutive, it has been shown to vary several fold in cells grown in the presence and absence of glucose.[26-28] Thus, it is not possible to exclude competition for enzyme forming molecules as a possible contributory factor to the glucose inhibitory effect, on the basis that the glucozymase is said to be constitutive.[22, 23] In any case, failure to find any differences in rate of glucose utilization by intact cells,[22] grown in the presence or absence of glucose may not be a reliable indication of the levels in the cells of enzymes concerned with glucose utilization.

Summary.—*Salmonella typhimurium*, strain LT2, (C^+dg^s) gives rise to mutants (C^-dg^s) which are unable to utilize glucose (or other carbohydrates), glycerol, or pyruvate as sole sources of carbon and energy for growth, although they still behave like the parent strain in being able to use various Krebs cycle compounds for this purpose. Although excellent growth of these mutants occurs in a mineral citrate medium, the addition of glucose severely inhibits growth, and glucose resistant (dg^r) mutants are isolated which are still unable to use glucose, glycerol, or pyruvate as sole carbon and energy sources for growth (C^-dg^{r-1}). By transduction with phage PLT 22, previously grown on the wild type (C^+dg^s), it was possible to convert C^-dg^{r-1} to C^+dg^{r-1}. A study of the wild type and these variants has demonstrated the following: the glucose inhibition is a ramification of the glucose-citrate diauxie phenomenon exhibited by the wild type, and mutation to glucose resistance is actually mutation to diauxie resistance, when specifically glucose is a member of a pair of compounds to which the wild type exhibits diauxie. The dg^{r-1} marker in an otherwise wild type genetic background acts as a modifier resulting in: the lowering of the growth rate specifically with glucose as substrate, and causing a two- to threefold increase in activity of an acid phosphatase. Glucose does not inhibit directly the penetration of citrate into the cell but rather the synthesis of some factor or factors required for citrate "permeation."

The author wishes to thank Mrs. Laura Ingraham and Mrs. Joanna Hadden for their able technical assistance.

* This investigation was supported in part by the Office of Naval Research and by research grants from the National Science Foundation and the National Institute of Allergy and Infectious Disease, U.S. Public Health Service. Reproduction in whole or in part is permitted for any purpose of the United States Government. Brief summaries of this work have appeared in *Genetics*, **42**, 368 (1957); Seventh International Congress for Microbiology (*Abstracts*), 21–22 (1958); and in the *Annual Report of the Biological Laboratory, Long Island Biological Association*, 1956–1957, 1957–1958. A major portion of this work was performed at the Biological Laboratory, Long Island Biological Association, while the author was on the staff there.

[1] Preliminary analysis indicates that the deficiency of the C^- mutants is apparently in the ability to fix CO_2 for the extra Krebs cycle synthesis of C4 dicarboxylic acids by means of a C3 plus C1 condensation. The details of these experiments will be reported elsewhere. The actual site of the deficiency is, however, not pertinent to the experiments reported here.

[2] Monod, J., *La Croissance des Cultures Bacteriennes* (Paris: Hermann et Cie, 1942).

[3] Zinder, N., and J. Lederberg, *J. Bacteriol.*, **64**, 679 (1952).

[4] In early experiments the above synthetic medium was found to be partially inhibitory when employed with glucose and many other compounds as carbon sources. The addition of citrate, any amino acid (*d* or *l*, 100 gamma/ml), nutrient broth carried over with the inoculum, or Versene, was effective in neutralizing the inhibition. None of these supplements changed essentially the differences noted between the various mutants studied. It should be noted that this inhibitory effect, probably caused by some heavy metal ion, was highly specific for *Salmonella typhimurium*, since strains of *Pseudomonas fluorescens* and *Escherichia coli* were not so affected.

[5] Hershey, A., and M. Chase, *J. Gen. Physiol.*, **36**, 39 (1952).

[6] Adams, M. H., in *Methods in Medical Research*, ed. J. H. Comroe (Chicago: The Year Book Publishers Inc., 1950), vol. **2**, p. 1.

[7] Fiske, C. H., and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

[8] Stadtman, E. R., C. D. Novelli, and F. Lipmann, *J. Biol. Chem.*, **191**, 365 (1951).

[9] Colowick, S. P., and H. M. Kalckar, *J. Biol. Chem.*, **148**, 117 (1943).

[10] Roe, J. H., J. H. Epstein, and N. P. Goldstein, *J. Biol. Chem.*, **178**, 839 (1949).

[11] Dische, Z., and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

[12] Grafflin, A. L., and S. Ochoa, *Biochem. et Biophys. Acta.*, **4**, 205 (1950).

[13] Garen, A., and N. D. Zinder, *Virology*, **1**, 347 (1955).

[14] The $C^{+}dg^{r}-1$ transductant employed in these experiments was kindly isolated for the author by Dr. Sheila Howarth.

[15] Davis, B. D., in *Enzymes: Units of Biological Structure and Function*, ed. O. H. Gaebler (Academic Press, New York, 1956), p. 509.

[16] Barett, J. T., A. D. Larson, and R. E. Kallio, *J. Bacteriol.*, **65**, 187 (1953).

[17] Rickenberg, H. V., G. H. Cohen, C. Buttin, and J. Monod, *Ann. inst. Pasteur*, **91**, 829 (1956).

[18] The author is indebted to Dr. N. Entner for performing the initial experiment dealing with the acid phosphatase.

[19] Pardee, A., F. Jacob, and J. Monod, *Compt. rend. acad. sci.*, **246**, 3125 (1958).

[20] Gale, E. F., *Bacteriological Reviews*, **7**, 139 (1944).

[21] Cohn, M., in *Enzymes: Units of Biological Structure and Function*, ed. O. H. Gaebler (Academic Press, New York, 1956), p. 41.

[22] Niedhardt, F. C., and B. Magasanik, *J. Bacteriol.*, **73**, 253 (1957).

[23] *Ibid.*, **73**, 260 (1957).

[24] Wagner, R. P., and H. K. Mitchell, in *Genetics and Metabolism* (New York: John Wiley & Sons, 1955).

[25] Entner, N., and E. Englesberg, *Nature*, **182**, 1808 (1958).

[26] Stephenson, M., and E. F. Gale, *Biochem. J.*, **31**, 1311 (1937).

[27] Spiegelman, S., and R. Dunn, *J. Gen. Physiol.*, **31**, 153 (1947).

[28] Scott, D. D. McNair, A. M. Pakoskey, and E. Chu, *Bacteriol Proc.*, **102** (1959).

---

*MUTATIONAL HETEROZYGOTES IN BACTERIOPHAGES**

By DAVID PRATT† AND GUNTHER S. STENT

VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated by W. M. Stanley, August 24, 1959

Approximately 2 per cent of the progeny in crosses of bacteriophages T2 or T4 are heterozygous for a given genetic locus. Upon their multiplication these heterozygotes segregate and give rise to homozygous progeny carrying only one or the other of the two parental alleles which entered the cross. This heterozygosis is only partial, since a phage heterozygous for one locus is most often homozygous for any other, unless the two loci in question are closely linked.[1, 2] Further study of the

genetic structure of these heterozygotes has suggested that they are formed at the time of the elementary genetic exchange by which recombinant phages arise.[3] Recombinational heterozygotes of this sort have also been observed in crosses of bacteriophages T1[4] and λ.[5] It is the purpose of this report to show that heterozygous phage particles arise also by *mutation* of the viral genome.

Experimental—Mottled plaques in mutated phage stocks: If a culture of *Escherichia coli* infected with the r^+ wild type of T2 phage is exposed to the mutagen 5-bromodeoxyuridine (BD) during intracellular phage growth, a great increase over the spontaneous frequency of rapid lysis r mutants is found among the phage progeny.[6] A minor fraction of such induced r mutants appears in *mottled plaques* containing phages of both r and r^+ genotypes. At the outset of the present experiments it was observed that if the exposure of the r^+ infected culture to BD is of only short duration, the mutagen being added just prior to maturation of the phages subsequently assayed, then, although the absolute frequency of r mutants is much lower than for longer exposures to BD, the proportion of the r mutants which appears in mottled plaques is high, often over 30 per cent. This high proportion of BD-induced r mutants appearing in mixed clones might reflect the fact that the phage genome is "diploid" and that the elementary mutational event occurs in only one of the two homologous genetic structures. In this case mutation would create a heterozygous virus carrying both r and r^+ alleles, which would segregate into homozygous r and r^+ phages during the first growth cycle on the assay plate. The appearance of mottled plaques is also susceptible to quite a different explanation. It is possible that mottled plaques arise from virus particles which were not yet mutated at the time of plating. For since the mutagen BD is extensively incorporated into the phage DNA,[7] BD-substituted r^+ phages might mutate to r on the assay plate, giving rise to mixed clones of r and r^+ individuals, i.e., to mottled plaques.

To decide whether mutational heterozygotes actually exist, it is necessary to eliminate the possibility that mottled plaques arise by mutation on the assay plate. This can be achieved by placing between the BD-grown phage stock and the assay plate a screen which lets through, i.e., permits to multiply, only those phages which have *already* mutated at the time of plating. In the present experiments the *reverse* mutation of r_{II} mutants[8] to the r^+ wild type has been examined, and strain K12(λ)‡ of *E. coli*, hereafter referred to as strain K, has been employed as the screen. For strain K does not support the multiplication of r_{II} phages but does allow the growth of the r^+ wild type[8] as well as of recombinational heterozygotes of the r^+/r_{II} type.[2]

Preparation and assay of mutated phage stocks: In this work an r_{II} mutant of bacteriophage T4 has been employed whose reverse mutation to the r^+ wild type is inducible by BD.[9, 10] This mutant forms r-type plaques on *E. coli* strain S‡ and does not grow on strain K. A stock of this mutant was grown which contained 10^{-7} r^+ revertants per r_{II} phage. This stock was used to infect *E. coli* strain R2‡ at a multiplicity of 2 phages per bacterium. For infection, R2 was grown to a titer of 10^8 cells/ml in a glucose-salts medium[5] and was centrifuged and resuspended, 5-fold concentrated, in phosphate buffer containing 20 μg/ml tryptophane. Phage adsorption was allowed to proceed in the buffer for 5 minutes at 37°, after which time the infected cells were centrifuged out of the adsorption mixture and re-

suspended in glucose-salts medium for incubation at 37°. BD was added to a concentration of 200 μg/ml after 10.5 minutes of incubation, i.e., a minute or two before the maturation of the first progeny phages. Aliquots of the infected bacteria were lysed with chloroform[11] at various times after addition of the mutagen. The phage progeny of the lysates were assayed on plates seeded with K and S bacteria respectively, the ratio of the infective titers obtained in the two assays indicating the frequency of r^+ revertants among the progeny.

Heterozygote test: To test also for the presence of any mutational heterozygotes the following "screening" was carried out. Prior to their plating on strain S, aliquots of the phage progeny were preadsorbed to K cells, at a multiplicity of 0.01 phages per bacterium. Cultures of strain K for preadsorption were grown in broth to a density of 10^8 cells/ml, concentrated by centrifugation to a density of 2.5×10^8 cells/ml in phosphate buffer, exposed to ultraviolet light for four minutes at 80 cm distance from a 15 watt germicidal lamp, and concentrated to 10^9 cells/ml in buffer containing 20 μg/ml tryptophane. Phage adsorption was allowed to proceed for 4 minutes, and any unadsorbed phage particles were then neutralized with antiserum. The infected bacteria were centrifuged out of the adsorption mixture, resuspended in buffer and finally plated on plates seeded with strain S. Under these conditions r_{II} phages, making up the bulk of the progeny populations, do not give rise to plaques, except for a "transmitted" fraction of about 10^{-4} which does succeed in myltiplying in the K cells to which it has been adsorbed and which then gives rise to pure r plaques on the assay plate. Each r^+ revertant among the progeny, on the other hand, gives rise to an r^+ plaque, while a heterozygous r^+/r_{II} revertant, if it is able to grow on K and segregate r^+ and r_{II} progeny, should produce a mottled plaque on the assay plate.

Frequency of heterozygotes: Table 1 shows the results of a number of such experiments. It can be seen there that depending on the length of exposure of the infected bacteria to BD before lysis, the proportion of r^+ revertants in the progeny increased by two to three orders of magnitude from the spontaneous input level of 10^{-7}. It is evident, further, that an important fraction of these induced r^+ revertants give rise to mottled plaques when plated on strain S after "screening" by preadsorption to strain K, the fraction being highest in the aliquots which received the shortest exposures to BD. Among the phage progeny grown in bacteria exposed to BD for only four or five minutes before lysis, 60 to 80 per cent of the r^+ revertants produced mottled plaques.

These results indicate that the frequent occurrence of mutant phages in mixed clones is not due to mutations on the assay plate but must represent instead the existence of *mutational heterozygotes.* The fact that the proportion of such heterozygotes among revertants decreases with the length of exposure to the mutagen then most probably reflects the continuous segregation during vegetative reproduction of *de novo* heterozygous mutants into homozygous r^+ revertants and r_{II} parental types. In preliminary experiments not presented here in detail it was found, in fact, that the proportion of heterozygotes among r^+ revertants drops to 5 to 10 per cent in r_{II} phage stocks which have been grown in the presence of BD for several cycles of infection and lysis.

It is possible that with even shorter exposures of the infected bacteria to BD than those employed here, the proportion of mutational heterozygotes might rise to 100

per cent, but the attendant low total frequency of induced revertants would make this difficult to demonstrate.

Some control experiments: A number of additional considerations would appear necessary before the existence of mutational heterozygotes can actually be accepted. First of all, it is conceivable that the mottled plaques found in the assays of Table 1 might reflect some nonrandomness in the adsorption of the progeny populations to the "screen" of K bacteria, so that even though the average multiplicity of infection in the preadsorption mixture was only 0.01 phages per cell, a significant proportion of the K cells infected with r^+ revertant phages was actually also infected with nonreverted r_{II} phages. Control experiments were therefore carried out in which

TABLE 1

MUTATIONAL HETEROZYGOTES AMONG r^+ REVERTANTS

Phage Preparation	Time of Lysis, Minutes after Addition of BD	Average Burst Size	Proportion of r^+ Revertants in Total Yield	Proportion of Heterozygotes among Revertants $\left(\frac{\text{mottled plaques}}{\text{mottled and } r^+}\right)$
Input T4r_{II}	..	..	10^{-7}	...
BD Experiment:				
1	4.5	0.9	13×10^{-5}	$0.61\left(\frac{207}{342}\right)$
	50	13	23×10^{-5}	$0.26\left(\frac{114}{442}\right)$
	4.5	1.7	3.8×10^{-5}	$0.71\left(\frac{399}{558}\right)$
	15	17	15×10^{-5}	$0.50\left(\frac{403}{799}\right)$
	3.5	0.2	5.2×10^{-5}	$0.77\left(\frac{259}{336}\right)$
	4.5	0.7	15×10^{-5}	$0.81\left(\frac{65}{80}\right)$
	5.5	1.1	11×10^{-5}	$0.70\left(\frac{153}{217}\right)$
	15	11	21×10^{-5}	$0.38\left(\frac{162}{422}\right)$
Non-BD r_{II}, r^+ Control Mixture:				
1	..	..	4.2×10^{-5}	$0.02\left(\frac{2}{107}\right)$
2	..	..	1.1×10^{-5}	$0.02\left(\frac{1}{58}\right)$

reconstruction mixtures of r_{II} and r^+ phages, each grown in the absence of BD, were adsorbed to strain K and plated on strain S under exactly the same conditions as in the heterozygote tests. The results of two such experiments are presented in Table 1, where it may be seen that in these controls the frequency of mottled plaques is no higher than that to be expected from the random probability of adsorbing more than a single phage per K cell.

Secondly, it might be thought that the origin of the heterozygotes could, after all, be recombinational, since in the vegetative phage pool of an infected bacterium in which a mutation occurred, mutant and nonmutant individuals could conceivably mate and generate recombinational heterozygotes. The very high frequency of heterozygotes among revertants makes this hypothesis extremely unlikely, however,

since the percentage of recombinational heterozygotes among revertants should not exceed the heterozygote frequency of 2 per cent normally encountered in T4 crosses.

Although r_{II} mutants infecting strain K do not give rise to mature, infective progeny phages, they apparently can proceed normally for the first 10 minutes of vegetative phage multiplication,[12] including the synthesis of phage DNA.[12, 13] A further objection may, therefore, be raised against the present heterozygote tests. For if a BD-labeled r_{II} phage infecting a K cell proceeds far enough in its multiplication to allow it to generate r^+ revertants, and if the belated appearance of r^+ genotypes allows the infected bacterium to produce progeny phages, then such a bacterium will give rise to a mottled plaque when plated on strain S. Should mottled plaques arise in this way, strain K would not be a suitable screen after all, and it would be a mistake to equate mottled plaques with mutational heterozygotes in the present tests. The following pair of control experiments shows, however, that mottled plaques cannot arise in this way from the particular T4r_{II} mutant employed in the present work.

The first of these control experiments was designed to test whether r^+ revertants are produced when r_{II} phages containing no BD in their DNA infect strain K in a BD-supplemented medium. For this purpose, a culture of K growing in DIFCO nutrient broth[11] containing 200 μg/ml BD was infected at a multiplicity of two phages per cell with the same stock of T4r_{II} employed in the previous experiments, containing a frequency of $10^{-7}r^+$ revertants. After 5 minutes for adsorption, the infected culture was diluted into BD-containing broth to give 4×10^6 infecting particles per ml, or a titer of 0.4 per ml of the background spontaneous r^+ revertants. Volumes of 0.5 ml of this dilution were distributed into 65 small tubes and incubated at 37° for 40 minutes, a time long enough for r^+-infected cells to yield progeny particles. The entire content of each tube was then plated on an assay plate seeded with strain K, to determine the proportion of tubes containing any r^+ phages, i.e. . , the fraction of infecting phages generating wild-type revertants. The result of this test was that the content of 35 of the tubes gave plaques on strain K, showing that the proportion of infecting particles generating any r^+ progeny was 3.8×10^{-7}. In a parallel experiment in which no BD had been added to the nutrient broth, 1.4×10^{-7} of the infecting particles gave rise to r^+ progeny. The rather slight effect of BD in stimulating reversions over the spontaneous background level presumably results from induced mutations in that small proportion of r_{II}-infected K bacteria from which infective progeny do manage to issue. It is apparent that if any BD-induced reversions occur during the abortive vegetative phage multiplication of r_{II} in strain K, then these do not in general lead to the appearance of any infective r^+ progeny.

It should be noted, however, that in this control experiment the DNA of the infecting r_{II} phages does not contain any BD, and hence the mutagen would not be in phage DNA until the start of DNA replication. In the heterozygote tests, the DNA of the r_{II} input phages does contain BD. It is possible that this already-incorporate mutagen induces the production of r^+ revertants at the very first replication act in strain K, hence soon enough to "rescue" the infective center. On the other hand, it is possible that mutagen incorporated only into replica DNA molecules cannot induce reverse mutations soon enough to permit synthesis of infective progeny in

r_{II}-infected K cells. The first of these control experiments does not, therefore, rigorously exclude the occurrence of r^+ reversions in K cells but shows that if such reversions occur they can only lead to infective revertant progeny if they take place at the very outset of vegetative growth.

A second type of control experiment was, therefore, carried out, designed to test whether BD-labeled r_{II} phages of the present mutant strain do, in fact, sport r^+ revertants during growth in the absence of an external supply of BD. The principle of this experiment is the following. If it is supposed that r^+ revertants do arise without external BD, then the number of these revertants should be greater if BD-labeled r_{II} phages infect bacteria of strain S than if K bacteria are similarly infected. For since strain S supports the *normal* multiplication of r_{II} phages, any reversions induced by the replicating BD-labeled parental DNA, whether induced early or late in vegetative growth, should give rise to infective r^+ progeny phages. On the other hand, as shown by the previous control experiment, only reversions occurring at the very outset of the abortive growth of r_{II} in strain K could possibly lead to infective r^+ progeny. Hence if a BD-labeled stock of r_{II} phage is preadsorbed to bacteria of either strain S or strain K before plating on agar seeded with strain K, then the number of infective centers forming plaques, ie., releasing any r^+ progeny, should be greater after preadsorption to S than after preadsorption to K.

For this experiment, a BD-labeled virus stock was grown in the following manner: 200 μg/ml BD was added to a culture of strain R2 growing in glucose-salts medium, 10.5 minutes after its infection with T4r_{II} phages, and progeny phages were harvested by chloroform lysis 5.5 minutes after addition of the mutagen. The ratio of the assays of this preparation on strains K and S indicated a frequency of $1.1 \times 10^{-4} r^+$ revertants, of which 60 per cent gave rise to mottled plaques in the heterozygote test. Aliquots of this BD-labeled stock were plated on strain K after preadsorption at low multiplicity to broth-grown, ultraviolet light-treated cells of either strain K or strain S. Plaques appearing in such platings represent the r^+ revertants originally present in the BD-labeled stock, and in addition any *de novo* r^+ revertants which can arise during growth in the cells of S or K to which the virus particles have been preadsorbed. If *de novo* revertants formed in K are in fact responsible for the mottled plaques in the heterozygote test, then 60 per cent of the total revertants in a K plating of this particular phage stock are of *de novo* origin. Since severalfold more of this class of revertants should be formed in S cells, then in this control experiment the total number of S cells releasing revertants should be much higher than the total number of K cells doing so.

The result of this control experiment is presented in Table 2, where it may be seen that the corrected titer of plaque-formers on strain K is no higher when the BD-labeled stock of T4r_{II} is preadsorbed to S bacteria before plating than when preadsorption is made to K bacteria. It seems likely, therefore, that the presence of BD in the DNA of the r_{II} mutant employed in this work does not induce reversions to r^+ with any appreciable frequency in the absence of BD from the growth medium. It would follow that the mottled plaques observed in the heterozygote tests cannot derive from mutations occurring during vegetative development of r_{II} mutants in the K bacteria employed as the "screen" but must really represent r^+/r_{II} heterozygotes already present in the BD-labeled phage stock.

Discussion—The principal facts observed in these experiments are two. First,

mutations induced by the addition of 5-bromodeoxyuridine (BD) to T4-infected bacteria result in heterozygous bacteriophage particles. Second, the proportion of mutants which are heterozygous declines with the length of time for which intracellular phage growth is allowed to proceed after addition of the mutagen.

These facts demonstrate that the T4 bacteriophage particle is "diploid," carrying the genetic information twice for a given locus. The molecular basis of this diploidy remains to be determined. One possibility is that the twofold genetic information

TABLE 2

THE EFFECT OF PREADSORPTION TO EITHER STRAIN S OR STRAIN K ON TITERS OF r^+ REVERTANTS IN A 5-BROMODEOXYURIDINE-LABELED T4rII STOCK

Phage Preparation	Preadsorption to: / Plating on:	Infective Centers per Milliliter: None / S	None / K	S / K	K / K
Control T4r^+, No BD		6.8×10^{10}	3.7×10^{10}	6.8×10^{10}	3.5×10^{10}
BD Preparation of T4rII		2.2×10^{8} (r plaques)	2.5×10^{4}	5.0×10^{4}	2.9×10^{4} (uncorrected) 5.5×10^{4} (corrected for efficiency of plating)*

* From the control platings it may be seen that T4r^+ plates more efficiently on K if preadsorbed to S than if preadsorbed to K. A correction factor for this difference in efficiency of plating, computed from the control r^+ platings, was applied to the revertant r^+ platings to permit direct comparison of the titers obtained by preadsorption to either S or K. The correction factor $= \frac{\text{control } r^+ \text{ titer, preadsorption to S}}{\text{control } r^+ \text{ titer, preadsorption to K}} = 1.9$.

for a locus is carried by two homologous DNA molecules, only one of which carries the mutated allele in a mutational heterozygote. There is, however, no evidence for homologous DNA molecules in bacteriophages. Furthermore, the results of genetic recombination experiments in phages can be interpreted on such a basis only if it is assumed that homologous molecules are coupled together in some special manner during vegetative multiplication in an infected bacterium. For if they were not coupled together, a large fraction of the progeny of a phage cross should be heterozygous for many genetic loci, which is contrary to the experimental finding of a low frequency of phages with a very limited partial heterozygosity.[1]

If, as seems most likely, a phage particle carries the information for a genetic locus in only *one* DNA molecule, a second possible explantation for the molecular basis of diploidy and mutational heterozygotes may be invoked, based on the mechanism of DNA replication and mutation proposed by Watson and Crick.[14] They envisioned that the two complimentary polynucleotide chains of a DNA molecule separate from each other for replication, each chain serving as a template for the synthesis of a new chain of complementary purine and pyrimidine base sequence. A mutation, from this point of view, is that rare event in which at one particular site in a replica chain a purine or pyrimidine base noncomplementary to the nucleotide in the parental chain happens to have been incorporated. The first double-stranded DNA molecule resulting from this mutational act should thus contain one nonmutated parental and one mutated replica chain, i.e., should be heterozygous for the mutant locus. Upon subsequent replication of this heterozygous molecule, the two complimentary chains would separate from each other to generate two homozygous daughter molecules, one mutant and the other nonmutant. In the case of the pool of vegetative viral DNA present in phage-infected bacteria, this segregation of heterozygous into

homozygous molecules at each replication cycle should cause a decrease in the proportion of heterozygote to total mutant molecules with prolonged multiplication in the presence of a mutagen.

The present findings are supported by the independent discoveries of mutational heterozygotes by Vielmetter and Wieder[15] and by Tessman.[16] These workers observed that a considerable proportion of the *r* mutants induced by *in vitro* treatment of free T2r^+ and T4r^+ phage particles with nitrous acid, appear in mottled plaques, i.e., in mixed *r* and r^+ clones. The interpretation that this heterozygosity reflects the two-stranded nature of the phage DNA is strengthened by Tessman's finding that no such mutational heterozygotes are observed upon nitrous acid mutagenesis in phage ϕX 174, a virus whose DNA is known to consist of only a single polynucleotide strand.[17]

If, as seems to be the case, heterozygosity of mutant bacteriophages does result from the existence of complementary mutant and non-mutant polynucleotide chains within a single phage DNA molecule, then it may be concluded that *both* strands of the DNA duplex of an infecting T2 or T4 bacteriophage actually replicate and give rise to functional progeny.

Summary—If bacteria infected with T2 or T4 bacteriophages are exposed to the mutagen 5-bromodeoxyuridine, some of the resulting mutant phages are heterozygous, in that they give rise to mixed clones of mutant and nonmutant progeny. The proportion of heterozygotes among mutants rises to at least 80 per cent when progeny phages are harvested very shortly after addition of the mutagen. This proportion is reduced by prolonged exposure of the infected bacteria to 5-bromodeoxyuridine. These facts can be interpreted to mean that the mutational heterozygosity of a phage particle resides in a double stranded DNA molecule which contains one mutant and one nonmutant polynucleotide chain.

One of us (D. P.) wishes to thank Dr. W. M. Stanley for the support and encouragement he has given during the tenure of a postdoctoral fellowship at the Virus Laboratory.

* Supported by a grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

† Postdoctoral Research Fellow of the National Science Foundation.

‡ The K12(λ) strain used in these experiments was K12-112, obtained from Dr. Franklin Stahl. Two other *E. coli* strains were used: $R2^{18}$ for preparation of BD-labeled T4rII stocks; S^{19} for platings and for preadsorption. Strain S is equivalent to the *E. coli* strain B used by Benzer[8] and should not be confused with the strain K12S which Benzer designates as S.

§ MI. medium, formula obtained from Dr. Matthew Meselson, contains per liter H_2O: 7 g Na_2HPO_4; 3 g KH_2PO_4; 1 g NH_4Cl; 1 ml 1*M* $MgSO_4$; 2 ml 25 per cent NaCl; 10 ml 20 per cent glucose; 0.2 ml 0.5 *M* $CaCl_2$; 0.3 ml 0.01 *M* $FeCl_3$; 10 ml 5 per cent vitamin-free casamino acids.

‖ Nutrient broth was used in this experiment after it was discovered that BD does not measurably induce mutation in T4r^+ phages multiplying in K cells in the glucose-salts medium, but that extensive mutation is induced in broth. Broth and the glucose-salts medium serve equally well for BD mutagenesis of T2 and T4 phages growing in strain R2 bacteria.

[1] Hershey, A. D., and M. Chase, *Cold Spring Harbor Symp. Quant. Biol.*, **16**, 471 (1951).

[2] Edgar, R. S., *Genetics*, **43**, 235 (1958).

[3] Levinthal, C., *Genetics*, **39**, 169 (1954).

[4] Trautner, T. A., *Z. Vererb.*, **89**, 264 (1958).

[5] Jacob, F., and E. L. Wollman, *Ann. Inst. Pasteur*, **87**, 653 (1954).

[6] Litman, R. M., and A. B. Pardee, *Nature*, **178**, 529 (1957).
[7] Dunn, D. B., and J. D. Smith, *Nature*, **174**, 305 (1954).
[8] Benzer, S., these PROCEEDINGS, **41**, 344 (1955).
[9] Benzer, S., and E. Freese, these PROCEEDINGS, **44**, 112 (1958).
[10] Freese, E., these PROCEEDINGS, **45**, 622 (1959).
[11] Séchaud, J., and E. Kellenberger, *Ann. Inst. Pasteur*, **90**, 102 (1956).
[12] Garen, A., personal communication.
[13] Uchida, H., personal communication.
[14] Watson, J. D., and F. H. C. Crick, *Cold Spring Harbor Symp. Quant. Biol.*, **18**, 123 (1953).
[15] Vielmetter, W., and C. M. Wieder, *Z. Naturf.*, **14b**, 312 (1959).
[16] Tessman, I., *Virology* (in press).
[17] Sinsheimer, R., *J. Mol. Biol.*, **1**, 43 (1959).
[18] Hershey, A. D., J. Dixon, and M. Chase, *J. Gen. Physiol.*, **36**, 777 (1953).
[19] Hershey, A. D., and R. Rotman, *Genetics*, **34**, 44 (1949).

*NON-RANDOM GENE DISTRIBUTION AMONG TOMATO CHROMOSOMES**

BY CHARLES M. RICK

UNIVERSITY OF CALIFORNIA, DAVIS

Communicated by Karl Sax, August 6, 1959

It was pointed out in 1956 by Rick and Butler[1] that the genes of the cultivated tomato, *Lycopersicon esculentum* Mill., do not seem to be randomly distributed among the 12 chromosomes. A satisfactory test for randomness could not be made at that time because certain chromosomes had been favored historically by having had a longer opportunity for detection of new linkages, and by having accumulated several marker genes apiece with a consequently greater length tested for new linkages. Notwithstanding these obstacles to the application of an exact probability treatment, they concluded ". . .it seems inevitable that this deviation from random distribution is real."

A better opportunity for testing random distribution has been afforded by a sample of 18 spontaneous seedling mutations accumulated here in the past six years. These were acquired from various sources in many different background genotypes. The only limitation imposed upon the sample is that of seedling expression—a relatively severe restriction for the reason that it favors mutations affecting pigmentation, stature, leaf shape, and disposition of hairs. Although it cannot be presently disproved that this restriction might affect chromosomal distribution of the genes concerned, such as effect is unlikely for the following considerations. First, as indicated below, the concentration of this sample on chromosome 2 is matched by the same tendency of mature-plant characters. Second, in other organisms that are better known genetically the genes affecting a particular organ seem to be distributed in the same fashion as the total known genotype.

Descriptions and linkage data have been published for all but two mutants, *La* and w_4, of this sample; references are indicated in Table 1. Each mutant was systematically screened for linkages by crosses with a series of marker genes, one per chromosome except that *Wo* was used as an additional marker for chromosome 2 in the tests with *ff* and *Mi* for the location of *yv* on chromosome 6. Linkages were

detected by standard F_2 tests, mostly in repulsion phase. For nearly all combinations a minimum of 400 plants was scored, thereby screening a linkage distance of about 40 units on either side of the marker gene. Tests were usually discontinued as soon as a significant indication of linkage was encountered.

TABLE 1

SUMMARY OF LINKAGE TESTS

	Chromosome and Tester Gene												
Mutant	1 *y*	2 d_1	3 *r*	4 *e*	5? *rp*	6 *c*	7 *tf*	8 l_1	9 *ah*	10 *H*	V a_1	? *La*	Reference
Cu	x*	L*	x	x	x	x	..	x	x	x	x	x	2
d_1^x	..	L	..	..	..	..	..	..	..	..	..	..	1
dv	x	L	x	..	..	x	x	x	..	x	x	..	3
ff	..	L†	..	..	x	x	x	..	x	x	x	x	2
Me	..	L	..	x	..	x	..	x	x	x	x	x	2
Wo^m	..	L	..	..	..	..	..	..	..	..	..	..	1
rv	x	x	x	x	..	x	..	x	x	x	x	x	2
sf	x	x	x	x	L	x	x	..	x	..	x	..	2
yv	x	x	x	x	x	L‡	..	x	x	x	x	x	4
tf	x	x	x	x	..	x	..	x	..	x	x	..	2
Fw	..	x	..	x	x	x	L	x	..	..	x	..	1
dl	x	x	x	..	x	x	x	L	..	x	x	x	3
ah	x	x	x	x	x	x	x	x	..	x	..	x	2
ag	..	..	..	..	x	..	..	..	..	L	..	..	1
gh	..	x	..	..	x	x	..	x	..	x	L	..	5
ht	x	x	x	x	x	x	x	..	x	..	x	..	2
La	x	x	x	x	x	x	x	x	x	x	x	..	..
w_4	?	x	x	..	x	x	x	x	..	x	x	..	..

* x signifies no indication of linkage. L signifies positive indication of linkage.
† Linkage of *ff* detected with *Wo*.
‡ Linkage of *yv* detected with *Mi*.

The results, summarized in Table 1, reveal the following distribution of the 18 mutants: chromosome 2: *Cu*, d_1^x *dv*, *ff*, *Me*, Wo^m; 5: *rv*, *sf*; 6: *yv*; 7: *tf*, *Fw*; 8: *dl*; 9: *ah*; 10: *ag*; group V (chromosome 11 or 12): *gh*; no linkage: *ht*, *La*, w_4.

A problem in the application of a probability treatment is posed by the three unlocated genes. For present purposes they are included in the analysis since they were tested against nearly all the markers. It is further assumed that neither is located on any of the tester chromosomes, thereby avoiding any bias in favor of chromosome 2. The probability that as many as 6 genes would be distributed to any single chromosome is given by the sum of the first 13 terms of the expansion of the binomial $(p + q)^{17}$, in which p, the probability of a gene residing on a specific chromosome, is $1/12$. The value obtained, 0.011, reveals a significant deviation from random distribution. If the data for *ff* (for which the discovery of a locus on chromosome 2 was favored by the use of 2 markers) are omitted, the probability calculated from the first 12 terms of $(p + q)^{16}$, 0.039, is still significant.

A non-random distribution of tomato genes has been suggested by other investigations. In a sample of 10 male-sterile mutants of a single horticultural variety, Pratt[6] assigned 2 to chromosome 2, three to group V, one each to chromosomes 1, 3, and 8, and two were not located. The deviation from randomness was not significant in this small sample, but the tendency is revealed of an accumulation on the already heavily laden chromosome 2. Currence,[7] testing the association between earliness and four markers on chromosome 2, found that most of the hereditary seasonal difference between parents could be ascribed to this chromosome. While the other chromosomes, which were not tested, doubtless exerted a genetic influence that was cancelled out, chromosome 2 wielded an astoundingly large share of genetic influence upon earliness.

A probability treatment of all located tomato genes is a vastly more difficult task for aforementioned reasons and because the chromosome map is based upon trisomic as well as ordinary linkage tests. The following data are therefore presented for what they might be worth without statistical tests. In the latest revised chromosome map of the tomato[8] (including the 15 genes of the present sample), 22 of the total of 87 placed genes lie on chromosome 2. Group V has 14 and chromosome 10 has 11. Thus, 25 per cent of the total lie on chromosome 2 and 54 per cent lie on the three chromosomes, 2, 10, and V.

Taking the distributions of the total and of the three restricted samples into account, the conclusion is unavoidable that mutant genes of the tomato seem to concentrate on certain chromosomes.

The following causes of the observed deviation might be considered: (1) The selected markers might not detect linkages on their respective chromosomes with equal likelihood. It would be a strange coincidence if the markers were equivalent in this respect. Their screening ability largely hinges upon their position. A few tomato chromosomes have been sufficiently explored to permit selection of favorably located markers (8, 10, V). The marker for chromosome 2, d_1, is less favorably situated, for, according to the latest revised map, only 2 genes have been detected in 11 units to its left, but 13 are known in the 66 units to its right. Position of the marker gene would therefore not tend to discriminate in favor of this chromosome except for the use of *Wo* to detect the linkage of *ff*.

(2) Tomato linkage groups probably differ in their total lengths. Visible pachytene lengths vary from 22.5 to 52.0 μ, but information concerning length of linkage maps is still very inadequate. Total pachytene length or achromatic length is weakly correlated with map length. By definition, chromosome 2 has the second longest pachytene length and in map length (77 units) is third longest. On the other hand, the longest linkage group must reside on one of the shortest chromosomes (No. 10) according to trisomic tests. Greater physical length might therefore account for at least part of the observed disproportion.

(3) Tomato genes might be disproportionately distributed regardless of chromosome length. The present linkage map for chromosome 2 offers support for this hypothesis, for at least four genes (*Wo*, *Me*, *Cu*, and ms_{10}) are tightly clustered near the middle of the group. Noteworthy is the presence of three dominant genes in this cluster, only 12 of the 87 mapped tomato genes being dominant.

Disproportionate gene distribution is nothing new in genetics. The high concentration of genes on the left end of the X chromosome of *Drosophila melanogaster* has been known for a long time. Another example is furnished by chromosome 9 of maize, the great majority of whose thirty-odd genes are concentrated in the short arm, comprising only one-third the total cytological length.[9]

Although the evidence for non-random gene distribution is abundant, convincing explanations are less readily found. For the tomato in particular, the question might be framed: "Why is one-fourth of the mutant genes concentrated on one of the 12 chromosomes?" The fact that chromosome 2 bears the only nucleolar organizer of the tomato set seems to have no particular bearing on this problem for accumulation of genes has not been found on the nucleolar chromosomes of maize,[10] barley,[11] or peas.[12]

The consistent grouping of duplicate genes in maize has been proposed by

Rhoades[9] as evidence of duplication of chromosomal segments, which would result in apparent unequal gene distribution. Within such groups recessive mutations would be masked by duplicates in some other part of the genome. For the tomato, to the contrary, the following evidence argues against the presence of extensive duplications: (1) intolerance of chromosomal unbalance; (2) sharply defined monogenic segregations and rarity of duplicate interactions; (3) failure of chromosome pairing in haploids.[1]

The concentration of genes in specific regions might reflect a tendency there toward increased mutability. Equally plausible might be selection for the grouping of genes (or reduction of crossingover between them) that function well together. Although the known crossover rates are too high to preserve large blocks together indefinitely, the tendency for genes to concentrate in specific regions, as in the vicinity of *Wo* on chromosome 2, might be advantageous. Mather[13] has called attention to the opposing selective values of linkage to preserve intact favorable groups of genes and recombination to increase variation. It is possible that, to compensate for the relatively high chromosome number of the tomato, the unequal distribution has evolved as a means of preserving balanced gene groups.

Whatever the cause or causes of this unusual condition, it has serious consequences upon the use of the tomato in genetic and breeding investigations. If the observed non-random distribution represents the status of the total germ plasm—a conclusion supported by Currence's[7] data—it would have a net effect of reducing chromosome number and increase in the probability of linkages. The transfer of monogenic characters from one genotype to another should ordinarily involve no serious difficulty, as modern experience in tomato breeding testifies. But with quantitative characters the problem of unfavorable linkages is intensified. The integration of several earliness genes in Currence's[7] example from one parent into the genotype of another without shifting many adjacent genes would present a formidable problem.

If the picture presented here is typical of spontaneous mutation, it would be of interest to learn how artificially induced mutations are distributed. Toward this end, work is now in progress to test the linkage relations of a series of new x-ray-induced tomato mutants produced by Stubbe.[14]

Summary.—In a sample of 18 spontaneous seedling mutants of the tomato, 6 were found to be located on chromosome 2. According to a binomial test, this distribution is non-random. For reasons presented the deviation does not likely result from the use of favorably located marker genes or from the presence of duplicated segments but probably reflects greater physical length and especially higher genetic activity of chromosome 2.

* Support for part of this work by National Science Foundation grant G-3355 is gratefully acknowledged.

[1] Rick, C. M., and L. Butler, *Adv. Genetics*, **8**, 267–382 (1956).
[2] Rick, C. M., and A. L. Harrison, *Jour. Hered.* (in press).
[3] Robinson, R. W., and C. M. Rick, *Jour. Hered.*, **45**, 241–247 (1954).
[4] Gilbert, J. C., *Tomato Genetics Coop. Report*, **8**, 15–17 (1958).
[5] Rick, C. M., A. E. Thompson, and O. Brauer, *Amer. Jour. Bot.*, **46**, 1–11 (1959).
[6] Pratt, D., Ph.D. Thesis, Univ. Calif. (1957).
[7] Currence, T. M., *Genetics*, **23**, 1–11 (1938).
[8] *Tomato Genetics Coop. Report*, **9**, (1959).

[9] Rhoades, M. M., *Amer. Nat.*, **85**, 105–110 (1951).
[10] Rhoades, M. M., *Corn and Corn Improvement* (New York: Academic Press 1955), Chap. 4.
[11] Robertson, D. W., G. A. Wiebe, and R. G. Shands, *Agron. Jour.*, **47**, 418–425 (1955).
[12] Lamprecht, H., *Agri. Hort. Genet.*, **16**, 38–48 (1958).
[13] Mather, K., *Jour. Genetics*, **43**, 309–336 (1942).
[14] Stubbe, H., *Die Kulturpflanze*, **5**, 190–220 (1957).

ON THE RADICAL OF A POSITIVE SEMIRING

BY SAMUEL BOURNE

UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated by Francis D. Murnaghan, August 28, 1959

W. Slowikowski and W. Zawadowski[4] defined a positive semiring $\mathfrak{S}$ to be a commutative semiring with zero and identity in which $1 + x$ has an inverse, for every $x \epsilon \mathfrak{S}$. They[4] defined the radical of a positive semiring $\mathfrak{S}$ to be the intersection of all maximal ideals of $\mathfrak{S}$. In his review of this paper of these authors, E. Hewitt[3] raised the following query: "It would be interesting to know the relation between the radical Rad $\mathfrak{S}$ and the Jacobson radical A introduced by Bourne."[1] We are now able to give the following answer:

In our paper on the semiradical of a semiring, Zassenhaus and I proved that *the semiradical $\sigma(S)$ of a semiring S is the intersection of its semimaximal semimodular left ideals.*[2] We recall the definition of a semimodular left ideal. *A closed left ideal L of a semiring S is called semimodular if there exist elements s_1, s_2 in the S such that $s_1 + u \not\equiv s_2 + u(L + \sigma(S))$ for $u \epsilon S$ and if for any element x of S there is an element y of S such that $x + xs_2 + y \equiv xs_1 + y(L)$*[2].

It is clear that if S possesses the elements O and 1, as is the case with a positive semiring, then every ideal is semimodular. We simply let $s_2 = 0$ and $s_1 = 1$ and semimaximality is merely maximality. Thus *the radical rad $\mathfrak{S}$ is the semiradical $\sigma(\mathfrak{S})$.*

[1] Bourne, S., "The Jacobson Radical of a Semiring," these PROCEEDINGS, **37**, p. 166 (1951).
[2] Bourne, S., and H. Zassenhaus, "On the Semiradical of a Semiring," these PROCEEDINGS, **44**, 907–914 (1958).
[3] Hewitt, E., *Math. Revs.*, **18**, p. 223 (1957).
[4] Slowikowski, W., and W. Zawadowski, "A generalization of maximal ideals method of Stone and Gelfand," *Fund. Math.*, **42**, 215–231 (1955).

*ON SUBMANIFOLDS OF CERTAIN FUNCTION SPACES**

By James Eells, Jr.

COLUMBIA UNIVERSITY

Communicated by Hassler Whitney, August 7, 1959

Let M be a smooth[1] manifold and A a submanifold of M. Fix a point m_0 of M, and let E_A denote the function space of paths on M which start at m_0 and end on A; we will specify below the precise nature of these paths and the specific structure of E_A.

The topological properties of E_A—in particular, those of its homology groups—have long been recognized as fundamental in algebraic topology and differential geometry, largely through the work of M. Morse and J-P Serre. The purpose of this note is to describe certain relations among the cohomology groups of such function spaces; for instance, we obtain an isomorphism relating the cohomology groups of E_A and those of its complement E_{M-A} in E_M. These results generalize to infinite dimensions the Alexander-Pontrjagin duality theory of the manifolds M,A; they also include as special cases certain known results in the cohomology of path spaces. The basic idea of the proof involves an infinite dimensional differentiable manifold structure[2] on E_M, study of the finite codimensional submanifold E_A in E_M, and standard theory[4] of its normal bundle.

1. Let $\mathfrak{I}(M)$ denote the tangent vector bundle of M with projection map π: $\mathfrak{I}(M) \to M$. For technical reasons it is convenient to introduce a smooth Riemann structure on M, possibly having special properties relative to the submanifold A; we endow $\mathfrak{I}(M)$ with its naturally induced Riemann structure. If u,v are vectors in the tangent space $M(m)$ of M at m, we let (u,v) denote their inner product and $|u|$ the length of u.

Suppose now that A is a regularly imbedded closed submanifold of M; then E_A consists of the totality of maps x of the closed unit interval $I = [0,1]$ into M such that

(1) $x(0) = m_0$, and $x(1)$ is a point of A;

(2) x is absolutely continuous in the metric of M; then its tangent vector $x'(t)$ exists for almost all t in I, and we require that

(3) the tangent map $x' : I \to \mathfrak{I}(M)$ is square integrable, in the sense that the Lebesgue integral, $\int_0^1 |x'(t)|^2 dt$, is finite.

It is known[2] that E_A *is a paracompact, infinite dimensional, smooth, Riemann manifold modeled on a separable real Hilbert space.* That means first of all that each point x in E_A has an associated tangent Hilbert space $E_A(x)$, the elements of which are the infinitesimal variations of x; if u,v are in $E_A(x)$, their inner product is

$$(u,v) = \int_0^1 (u'(t),v'(t))dt. \tag{1}$$

The differentiable structure in E_A is based on a theory of differentiation in Hilbert spaces, and uses the fact that each tangent space $E_A(x)$ is complete. The inner product (1) defines a smooth Riemann structure on E_A. See[2, 4] for more details and examples.

2. Suppose that B is a regularly imbedded closed submanifold of M which contains A. Let r be the codimension of A in B; i.e., for any point m in A the

tangent space $A(m)$ is a subspace of $B(m)$ such that $r = \dim (B(m)/A(m))$. We assume that r is positive, which ensures that A is a proper submanifold of B. It is easy to see that for any path x in E_A the tangent space $E_A(x)$ is a closed subspace of $E_B(x)$ and that $r = \dim (E_B(x)/E_A(x))$; otherwise said, *E_A is an r-codimensional submanifold of E_B*. Thus we can use the Riemann structure in E_B to define the normal r-dimensional vector bundle W over E_A; let $\mathfrak{F}$ denote the local system[3] of integers determined by W.

THEOREM. *For each dimension i there is a canonical isomorphism*

$$\theta: H^{i-r}(E_A; \mathfrak{F}) \to H^i(E_B, E_{B-A}),$$

where the groups are singular cohomology (absolute and relative) groups with local and integer coefficients, respectively.

The first step of the proof is to construct the Gysin-Thom[3] isomorphism ϕ: $H^{i-r}(E_A; \mathfrak{F}) \to H^i(W, W_0)$, where W_0 is the subspace of W consisting of the nonzero vectors. We next apply a theory of geodesics in the function space manifold E_B to find neighborhoods V of the zero section of W and U of E_A in E_B for which the identification of short geodesics and short tangent vectors provides a homeomorphism $g: U \to V$. Thirdly, we note that the inclusion maps $j: (V, V \cap W_0) \to (W, W_0)$ and k: $(U, U - E_A) \to (E_B, E_B - E_A)$ are excisions. Composing the induced cohomology isomorphisms, we set $\theta = k^{*-1} \cdot g^* \cdot j^* \cdot \phi$:

$$\begin{array}{ccccc} H^{i-r}(E_A; \mathfrak{F}) & \xrightarrow{\phi} & H^i(W, W_0) & \xrightarrow{j^*} & H^i(V, V \cap W_0) \\ & & & & \downarrow g^* \\ & & H^i(E_B, E_{B-A}) & \xrightarrow{k^*} & H^i(U, U - E_A) \end{array}$$

3. If we combine the cohomology sequence of the pair (E_B, E_{B-A}) with the Theorem, we obtain the

COROLLARY. *There is an exact sequence*

$$\ldots \xrightarrow{\lambda} H^i(E_{B-A}) \leftarrow H^i(E_B) \leftarrow H^{i-r}(E_A; \mathfrak{F}) \xrightarrow{\lambda} H^{i-1}(E_{B-A}) \leftarrow \ldots$$

COROLLARY. *Suppose that $H^q(E_B) = H^{q-1}(E_B) = 0$ for some dimension q. If δ denotes the connecting homomorphism of the cohomology sequence of (E_B, E_{B-A}), then we have an isomorphism*

$$\lambda = \theta^{-1} \cdot \delta: \; H^{q-1}(E_{B-A}) \to H^{q-r}(E_A; \mathfrak{F}).$$

For example, *if we take $B = M$, then E_B is contractible, and λ is an isomorphism for all $q > 1$.*

4. *Examples:* (a) If M is Euclidean space, then any closed submanifold B in M has the same homotopy type as E_B. In this case the above results reduce to known consequences of the Alexander-Pontrajagin duality of the pair (B, A).

(b) If M is arbitrary, B is an r-sphere ($r \geq 2$) and A is a point, then E_{B-A} has the same homotopy type as E_A; furthermore, the local system $\mathfrak{F}$ is simple. The exact sequence in 3 becomes the Wang sequence of the Serre fibration[5] p: $E_B \to B$, defined by $p(x) = x(1)$.

(c) To illustrate the use of the Theorem let us recover the periodicity relations for the cohomology groups of the loop space of complex projective n-space P_n. Consider the usual imbeddings $P_0 \subset P_1 \subset \ldots \subset P_n$; then $P_n - P_{n-1}$ is an open cell,

whence $E_{P_n-P_{n-1}}$ has the homotopy type of E_{P_0}. Since P_{n-1} has codimension 2 in P_n, we obtain an isomorphism λ: $H^{i-1}(E_{P_0}) \approx H^{i-2}(E_{P_n-1})$ for all $i > 1$. On the other hand, $E_{P_n-P_0}$ has the homotopy type of E_{P_n-1}, whence another application of the theorem gives an isomorphism λ: $H^{i-1}(E_{P_n-1}) \approx H^{i-2n}(E_{P_0})$ for all $i > 1$. Combining these we find $H^i(E_{P_0}) \approx H^{i-2n}(E_{P_0})$ for all $i > 1$.

We remark that this method of computation applies to many other examples of loop spaces; e.g., those of the spheres, the Cayley plane, and the manifolds of Wu-Dold. More involved applications will be presented elsewhere.

* Research partially supported by the Office of Naval Research.

[1] Precisely, M is paracompact, connected, infinitely differentiable, finite dimensional manifold.

[2] Eells, J., *On the Geometry of Function Spaces*, Symposium Internacional de Topologia Algebraica (Univ. Nac. Aut. Mexico), 303–308 (1958).

[3] Thom, R., *Espaces fibrés en sphères et carrés de Steenrod, Ann. Sci. École Norm. Sup.*, **69**, 109–182 (1952).

[4] Eells, J., *A Class of Smooth Bundles over a Manifold* (in preparation).

[5] Serre, J. P., *Homologie singulière des espaces fibrés, Ann. Math.*, **54**, 425–505 (1951).

---

*EXTERIOR DIFFERENTIAL SYSTEMS AND ELLIPTIC EQUATIONS**

BY BRUCE L. REINHART AND SHLOMO STERNBERG

RIAS, UNIVERSITY OF MARYLAND, AND HARVARD UNIVERSITY

Communicated by S. Lefschetz, August 7, 1959

The aim of this paper is to exhibit a relationship between elliptic partial differential equations and exterior differential systems, which is such as to suggest the possibility of an nonanalytic theory of exterior systems in involution.

Let $x^1, \ldots, x^n$ be independent variables, $u^1, \ldots, u^N$ dependent variables, and $u^\beta_{i_1 \ldots i_k}$ the partial derivatives of u^β. Let $\{S_\alpha\}$ $\{t_\beta\}$ be two sequences of N integers such that $\max S_\alpha = 0$, and let $r_{\alpha\beta} = S_\alpha + t_\beta$. Consider the system of partial differential equations:

$$(F) \qquad F^\alpha(x^i, u^\beta, \ldots, u^\beta_{i_1 \ldots i_r}) = 0 \begin{cases} i, i_\nu = 1, \ldots, n \\ \alpha, \beta = 1, \ldots, N \\ r = r_{\alpha\beta} \end{cases}$$

Considering all the symbols in parentheses as independent variables, let

$$l^{\alpha\beta}_{i_1 \ldots i_r} = \partial F^\alpha / \partial u^\beta_{i_1 \ldots i_r}.$$

Then $dF_\alpha = l^{\alpha\beta}_{i_1 \ldots i_r}\, du^\beta_{i_1 \ldots i_r} + \ldots$

The system (F) is called elliptic (in the sense of Douglis-Nirenberg[1]) if

$$\det \left| \left| l^{\alpha\beta}_{i_1 \ldots i_r} \xi^{i_1} \ldots \xi^{i_r} \right| \right| > 0.$$

for real vectors (ξ) different from 0.

Let us now define the exterior differential system (G) associated to (F). First remove the system (F) all equations F^α for which $S_\alpha < 0$, and replace each by the set of all of its derived equations of order $-S_\alpha$. Denote the equations of the new system by $G^\alpha = 0$, $\alpha = 1, \ldots, M \geq N$. In the space E of variables

$$(x^i, u^\beta, \ldots, u^\beta_{i_1 \ldots i_t}) \qquad t = t_\beta$$

consider the ideal generated by

$$\begin{cases} dG^\alpha \\ du^\beta_{i_1 \ldots i_m} - \sum_j u^\beta_{i_1 \ldots i_m j} dx^j \qquad 0 \leq m < t_\beta \\ \sum_j du^\beta_{i_1 \ldots i_{m-1} j} \wedge dx^j \end{cases}$$

in the ring of all differential forms. This ideal is the system (G).

If $X_1, \ldots, X_p$ are independent vectors tangent to E at some given point e, a p plane $(e, X_1, \ldots, X_p)$ is said to be integral if

(i) e satisfies the equations $G^\alpha = 0$.

(ii) Each vector X_i annihilates the one forms of (G).

(iii) Each pair (X_i, X_j) annihilates the two forms of (G).

Let $I_x{}^p$ denote the set of all integral p planes on which p of the variables $(x^1, \ldots, x^n)$ are independent.

The polar equations of a p plane $(e, X_1, \ldots, X_p)$ are the linear equations which express the condition that a vector Y at e shall generate with $X_1, \ldots, X_p$ an integral plane of (G). The system (G) will be said to be globally in involution[2] if the rank of the polar system is constant over $I_x{}^p$ for each p, $0 \leq p < n$.

THEOREM. *If the system (F) of partial differential equations is elliptic, then the associated exterior differential system (G) is globally in involution.*

By a remark of M. Hausner and Sternberg (unpublished) the second order parabolic, hyperbolic, and ultrahyperbolic equations all give rise to exterior systems which are not in involution. Hence, one is led to conjecture some sort of equivalence between the notions of ellipticity and involution.

* This work was supported in part by the Air Force Office of Scientific Research Contract No. AF 49(638)-382.

[1] Douglis, A., and L. Nirenberg, "Interior Estimates for Elliptic Systems of Partial Differential Equations," *Comm. Pure Appl. Math.*, **8**, 503–538 (1955).

[2] This is a global form of the notion of involution defined in Cartain, *Les systèmes différentielles extérieures et leurs applications geometriques* (Paris: Hermann, 1945).

---

*POLYNOMIALS OF BEST APPROXIMATION ON AN INTERVAL**

BY J. L. WALSH AND T. S. MOTZKIN

HARVARD UNIVERSITY AND UNIVERSITY OF CALIFORNIA

Communicated July 20, 1959

The present writers have recently[1,2] studied juxtapolynomials and their relation to polynomials of best approximation to a given function on a real finite point set, especially with reference to oscillation properties. The object of the present note is to indicate, partly without proof, new results on the analogous problems of approximation to a given continuous function on a closed finite interval E.

Let the function $f(x)$ be continuous on E. The polynomial $q_n(x) \equiv b_0x^n + b_1x^{n-1} + \ldots + b_n$ of degree n is said to be a *closer* polynomial to $f(x)$ on E than the polynomial $p_n(x) \equiv a_0x^n + a_1x^{n-1} + \ldots + a_n$ of degree n provided $q_n(x) \not\equiv p_n(x)$ and provided

$$|f(x) - q_n(x)| < |f(x) - p_n(x)| \text{ on } E \text{ where } f(x) - p_n(x) \neq 0, \qquad (1)$$

$$f(x) - q_n(x) = f(x) - p_n(x) \text{ on } E \text{ where } f(x) - p_n(x) = 0. \tag{2}$$

If no closer polynomial $a_n(x)$ exists, $p_n(x)$ is a *juxtapolynomial* to $f(x)$ on E. A polynomial $p_n(x)$ of best approximation of $f(x)$ on E in the sense that (among all polynomials of given degree n) it minimizes either of the deviations

$$\int_E |f(x) - p_n(x)|^p dx, \qquad p > 0, \tag{3}$$

$$\max [|f(x) - p_n(x)|, x \text{ on } E], \tag{4}$$

is clearly a juxtapolynomial to $f(x)$ on E of degree n.

Let the function $\varphi(x)$ be continuous on E with $\varphi(x_0) = 0$, and let λ be the largest integer for which with some $\delta(> 0)$ and some $A(> 0)$ we have

$$\frac{|\varphi(x)|}{|x - x_0|^\lambda} \leq A < \infty, \qquad 0 < |x - x_0| < \delta, \qquad x \text{ on } E; \tag{5}$$

we say that $\varphi(x)$ has a zero of *lower order or multiplicity* λ at $x = x_0$. If (5) is valid for no positive integer, we set $\lambda = 0$; if (5) is valid for all positive integers we set $\lambda = \infty$. We establish

LEMMA 1. *If $f(x)$ is continuous on the closed finite interval E, and if $q_n(x)$ is a closer polynomial (of degree n) to $f(x)$ on E than $p_n(x)$, then $p_n(x) - q_n(x)$ has a zero of order at least λ wherever $f(x) - p_n(x)$ has a zero of lower order λ on E.*

By (1), (2), and (5) we have in some neighborhood of any zero x_0 of $f - p_n$ on E

$$|p_n - q_n| \leq |f - q_n| + |f - p_n| \leq 2|f - p_n| \leq 2A|x - x_0|^\lambda,$$

which implies the conclusion. We can state at once

THEOREM 1. *If $f(x)$ and E satisfy the conditions of Lemma 1, and if $p_n(x)$ is a polynomial of degree n such that $f(x) - p_n(x)$ has zeros of total lower multiplicity greater than n on E, then $p_n(x)$ is a juxtapolynomial to $f(x)$ on E.*

Indeed, if $q_n(x)$ is a closer polynomial to $f(x)$ on E than $p_n(x)$, it follows from Lemma 1 that $p_n(x) - q_n(x)$ has zeros of total order at least $n + 1$ on E, so $q_n(x) \equiv p_n(x)$.

If the function $\varphi(x)$ is continuous on the interval E with $\varphi(x_0) = 0$, and if μ is the smallest integer for which we have with some $\delta(> 0)$ and some m

$$m\varphi(x) \cdot (x - x_0)^\mu > 0, \qquad 0 < |x - x_0| < \delta, \qquad x \text{ on } E, \tag{6}$$

$$|\varphi(x)| > |m(x - x_0)^\mu| > 0, \qquad 0 < |x - x_0| < \delta, \qquad x \text{ on } E, \tag{7}$$

we say that $\varphi(x)$ has a zero of *upper order or multiplicity* μ at $x = x_0$. If no finite μ exists (e.g., if x_0 is not an isolated zero of $\varphi(x)$) we set $\mu = \infty$. As an illustration of upper order, we note that the function $\varphi(x) \equiv |x|$ has a zero of upper order two at $x = 0$. The definition of upper order requires that $\varphi(x)$ and $m(x - x_0)^\mu$ have the same algebraic sign in $0 < |x - x_0| < \delta$.

An immediate consequence of (6) and (7) is

$$|\varphi(x) - m(x - x_0)^\mu| < |\varphi(x)|, \qquad 0 < |x - x_0| < \delta, \qquad x \text{ on } E. \tag{8}$$

We remark that *for a zero of any function $\varphi(x)$, there is valid the inequality*

$$\text{lower order } \lambda \leq \text{upper order } \mu. \tag{9}$$

Indeed we have from (7)

$$|\varphi(x)/(x - x_0)^{\mu+1}| > |m/(x - x_0)|,$$

so $|\varphi(x)/(x - x_0)^{\mu+1}| \leq A$ is impossible. Then by (5) we have $\lambda < \mu + 1$, which is (9).

In view of (9), a strengthening of Lemma 1 is

LEMMA 2. *If $f(x)$ is continuous on the closed finite interval E, and if $q_n(x)$ is a closer polynomial (of degree n) to $f(x)$ on E than $p_n(x)$, then $p_n(x) - q_n(x)$ has a zero of order at least μ wherever $f(x) - p_n(x)$ has a zero of upper order μ on E.*

Inequalities (1) and (2) imply for each x on E that unless $f(x) = p_n(x) = q_n(x)$, the value $f(x)$ is nearer to $q_n(x)$ than to $p_n(x)$, so $|f(x) - p_n(x)| > |q_n(x) - p_n(x)|/2$, and $f(x) - p_n(x)$ has the same algebraic sign as $q_n(x) - p_n(x)$. Lemma 2 follows now by (6) and (7).

Concerning upper multiplicity we prove a stronger result than Theorem 1:

THEOREM 2. *A necessary and sufficient condition that the polynomial $p_n(x)$ of degree n be a juxtapolynomial to the continuous function $f(x)$ on the interval E is that $\varphi(x) \equiv f(x) - p_n(x)$ have on E zeros of total upper multiplicity greater than n.*

As Theorem 1 follows from Lemma 1, the sufficiency of the condition here follows from Lemma 2.

Conversely, if this condition is not satisfied, there exists a polynomial $q_n(x)$ of degree n, having zeros of total multiplicity not greater than n, which on E has the same algebraic sign as $\varphi(x)$, and which has at each zero of $\varphi(x)$ on E a zero whose multiplicity is the upper multiplicity of the zero of $\varphi(x)$. Indeed, if $q_n^0(x)$ has the same zeros on E as $\varphi(x)$ and with the same upper multiplicities, either $q_n^0(x)$ or $-q_n^0(x)$ satisfies the requirements. For sufficiently small ϵ (> 0) we have (compare (8))

$$|\varphi(x) - \epsilon q_n(x)| < |\varphi(x)| \tag{10}$$

in each of mutually disjoint neighborhoods $0 < |x - x_k| < \delta_k$ of the zeros x_k of $\varphi(x)$ on E. In E outside of these neighborhoods we have $|\varphi(x)| \geq \Delta(> 0)$, sig $q_n(x) = \text{sig } \varphi(x)$, so (10) is also valid for sufficiently small ϵ, and $p_n(x)$ is not a juxtapolynomial to $f(x)$ on E. Theorem 2 is established.

Theorems 1 and 2 clearly apply to the polynomials of given degree which minimize the deviations (3) and (4).

If $f(x)$ is itself a polynomial or other function of class C^∞ of the real variable x on E, upper and lower order are identical with the usual order of a zero, so a necessary and sufficient condition that a polynomial $p_n(x)$ of degree n be a juxtapolynomial to $f(x)$ on E is that $f(x) - p_n(x)$ have on E zeros of total order greater than n.

We turn now to the oscillation properties of polynomials which minimize the deviation (3); here the value of p has considerable influence. Jackson has proved[3]

THEOREM 3. *If the function $f(x)$ is continuous on the closed bounded interval E, the polynomial $p_n(x)$ of degree n which minimizes (3) with $p > 1$ is either identical to $f(x)$ on E or is such that $f(x) - p_n(x)$ has at least $n + 1$ strong sign changes on E.*

We omit the proof, which is similar to that of Theorem 4 below.

Here the term $n + 1$ *(strong) sign changes* of a function $\varphi(x)$ on E indicates that there exist $n + 2$ points x_k of E such that $(x_1 < x_2 < \ldots)$ either

$$\varphi(x_1) > 0, \qquad \varphi(x_2) < 0, \qquad \varphi(x_3) > 0, \ldots,$$

or the reverse inequalities hold. However, $\varphi(x)$ has $n+1$ *weak sign changes* on E if for the same points x_k we have either

$$\varphi(x_1) \geq 0, \qquad \varphi(x_2) \leq 0, \qquad \varphi(x_3) \geq 0, \ldots,$$

or the reverse inequalities. Jackson has established[4] too

THEOREM 4. *If $f(x)$ is continuous on the interval E: $0 \leq x \leq 1$, and if $p_n(x)$ is a polynomial of degree n which minimizes (3) with $p = 1$, then $f(x) - p_n(x)$ either has a total of at least $n+1$ strong sign changes on E or vanishes identically on a subset of E of positive measure.*

If Theorem 4 is false, there exists a polynomial $q_n(x)$ which vanishes in the strong sign changes of $f(x) - p_n(x)$ and only there on E, and where different from zero on E has weakly the same algebraic sign as $f(x) - p_n(x)$. We shall study $f(x) - p_n(x) - \epsilon q_n(x)$, $\epsilon > 0$, as comparison function. Thanks to the continuity of $f(x)$ and $p_n(x)$, we may write ($\delta > 0$)

$$\text{meas } [|f - p_n| \leq \delta] \to 0 \text{ as } \delta \to 0; \tag{11}$$

of course only points of E are included in this notation. If E_1 denotes the subset of E on which

$$|\epsilon q_n| \leq |f - p_n|, \tag{12}$$

on $E - E_1$ we have

$$|f - p_n| < |\epsilon q_n| \leq \epsilon M, \tag{13}$$

where $|q_n(x)| \leq M$ on E. By (11) there follows $\eta = \eta(\epsilon) = \text{meas } [E - E_1] \to 0$ as $\epsilon \to 0$. On $E - E_1$ we deduce from (13)

$$\begin{aligned} |f - p_n - \epsilon q_n| &\leq |\epsilon q_n| \leq \epsilon M, \\ \textstyle\int_{E-E_1} |f - p_n - \epsilon q_n|\, dx &\leq \epsilon\eta M. \end{aligned} \tag{14}$$

Inequality (12) now yields

$$\begin{aligned} \textstyle\int_{E_1} |f - p_n|\, dx - \int_{E_1} |f - p_n - \epsilon q_n|\, dx &= \epsilon \textstyle\int_{E_1} |q_n|\, dx \\ &= \epsilon \textstyle\int_E |q_n|\, dx - \epsilon \int_{E-E_1} |q_n|\, dx \\ &\geq \epsilon \textstyle\int_E |q_n|\, dx - \epsilon\eta M, \end{aligned}$$

and finally by (14)

$$\textstyle\int_E |f - p_n|\, dx - \int_E |f - p_n - \epsilon q_n|\, dx \geq \epsilon \int_E |q_n|\, dx - 2\epsilon\eta M; \tag{15}$$

the last member is positive for sufficiently small ϵ, in contradiction to the extremal property of $p_n(x)$. This contradiction completes the proof.

Jackson has also proved[4]

THEOREM 5. *Under the conditions of Theorem 4 the extremal polynomial $p_n(x)$ is unique.*

We suppose there exist two distinct extremal polynomials $p_n(x)$ and $q_n(x)$, and shall reach a contradiction. We remark that the algebraic inequality

$$\left| f - \frac{p_n + q_n}{2} \right| \leq \frac{1}{2} |f - p_n| + \frac{1}{2} |f - q_n|, \tag{16}$$

valid for all values of the arguments, together with the strong inequality

$$\left| f - \frac{p_n + q_n}{2} \right| < \frac{1}{2} |f - p_n| + \frac{1}{2} |f - q_n|,$$

valid if f lies between p_n and q_n, and where of course $r_n \equiv (p_n + q_n)/2$ is a competing polynomial of degree n, shows that *f cannot lie between p_n and q_n at even a single point of E*, since the functions involved are continuous.

The polynomial r_n is extremal, by (16), and by Theorem 4 the function $f - r_n$ must have at least $n + 1$ distinct weak sign changes, at each of which (by the remark already made) we have $f - p_n = f - q_n = 0$. Thus $p_n(x) - q_n(x)$ has at least $n + 1$ distinct zeros and vanishes identically.

The present proof of Theorem 5, including Theorem 4, is much simpler and shorter than that of Jackson.

In the whole class of polynomials $Q_k(x)$ of degree k (with $Q_k(x) \not\equiv 0$) there exists at least one element for which the ratio

$$\int_0^1 |Q_k(x)|\, dx / \max[|Q_k(x)| \text{ on } E] \tag{17}$$

has a minimum value ρ_k, as follows easily by the Montel theory of normal families of functions. With this notation, Theorem 4 can be sharpened as follows:

THEOREM 6. *Under the hypothesis of Theorem 4, if $f(x) - p_n(x)$ has precisely $k(< n + 1)$ strong sign changes on E, it must vanish identically on a subset of E of measure $\geq \rho_k/2$.*

The proof of Theorem 6 is a modification of that of Theorem 4, where we now define a polynomial $q_k(x)$ of degree k which vanishes in the strong sign changes of $f - p_n$ on E, and only then on E, and where different from zero on E has weakly the same algebraic sign as $f - p_n$. If we set $\mu = \text{meas } [|f - p_n| = 0]$, relation (11) is to be replaced by

$$\text{meas } [|f - p_n| \leq \delta] \to \mu \qquad \text{as } \delta \to 0, \tag{18}$$

and we have also $n(\epsilon) \to \mu$ as $\epsilon \to 0$. Inequality (15) now follows as before, with q_n replaced by q_k, and contradicts the extremality of $p_n(x)$ unless we have

$$\int_E |q_k|\, dx - 2\mu M \leq 0, \qquad \mu \geq \rho_k/2.$$

The constant ρ_k can be determined in simple cases; we have $\rho_0 = 1$, $\rho_1 = 2^{1/2} - 1$.

Jackson, in his proof of Theorem 4, asserts[4] the existence of a positive number which if $f(x)$ does not have $n + 1$ strong sign changes must be exceeded by meas$[f - p_n = 0]$, but does not define the constant (here $\rho_n/2$) nor does he indicate the dependence of the constant on k if (as in Theorem 6) the number of strong sign changes of $f(x) - p_n(x)$ is $k(< n + 1)$. Jackson also states[4] that $f(x) - p_n(x)$ must vanish identically on a *set of intervals the sum of whose lengths* is greater than some positive constant m. This is not correct, as is indicated by the following counterexample. Let E_0 be a closed proper subset of E: $0 \leq x \leq 1$ of measure greater than one-half which contains no interval; for example E_0 may be a suitably chosen Cantor set. The function $f(x)$ defined as the distance from x to E_0 is non-negative throughout E and zero on E. For $n = 0$ the polynomial $p_0(x) = 0$ is extremal, as may be seen from the forward derivative

$$\frac{d}{dc} \int_0^1 |f(x) - c|\, dx = \text{meas } [f(x) \leq c] - \text{meas } [f(x) > c], \tag{19}$$

which is positive for $0 \leq c < \max f(x)$.

We postpone to another occasion the proof of

THEOREM 7. *Let the function $f(x)$ be of class C^∞ on a closed bounded interval E, and let $p_n(x)$ ($\not\equiv f(x)$ on E) be a polynomial of degree n which for some p, $0 < p < 1$, minimizes (3). Let $f(x) - p_n(x)$ have zeros of respective multiplicities κ_j, $1 \leq j \leq r$, and let κ_j^* be the smallest integer $\geq [(1 - p)\ \kappa_j]$ and which for zeros interior to E is also of the same parity as κ_j. Then we have*

$$\Sigma \kappa_j^* > n. \tag{20}$$

As an illustration here, suppose all κ_j are unity; at each end point of E we have $\kappa_j^* = 0$, so (20) is precisely the condition that the number of strong sign-changes of $f - p_n$ be greater than n.

The conditions developed in Theorems 3, 6, and 7 are necessary but in the judgment of the writers (which is still to be confirmed) are close to sufficient.

There are numerous differences between approximation (*i*) on a closed bounded interval E and[1,2] (*ii*) on a real finite set E_m. In (*ii*) every juxtapolynomial is extremal with $p = 1$ and suitable weight function, but not in (*i*), as is shown by the example $f(x) \equiv x^2$, $n = 0$, E: $0 \leq x \leq 1$; compare (19). In (*i*) the conditions derived become progressively stronger for juxtapolynomials and extremal polynomials with increasing p, namely $0 < p < 1$, $p = 1$, $p > 1$; in (*ii*) (for suitable weight function) the conditions are weakest for $p = 1$, stronger for $0 < p < 1$, still stronger for $p > 1$. In (*ii*) the conditions are independent of p, $0 < p < 1$, but not in (*i*). In (*i*) the extremal polynomial for $p = 1$ is unique, but not necessarily in (*ii*).

The present writers plan to continue this study, by considering further both necessary and sufficient conditions for best approximation to functions not necessarily continuous, using weight functions and more general real point sets.

* Research supported (in part) by the Office of Naval Research, U.S. Navy, and by the Office of Scientific Research, Air Research and Development Command. Reproduction in whole or in part is permitted for any purpose of the United States Government.

[1] Motzkin, T. S., and J. L. Walsh, these PROCEEDINGS, **43**, 845–846 (1957).

[2] *Ibid.*, *Trans. Amer. Math. Soc.*, **91**, 231–245 (1959).

[3] Jackson, D., *Trans. Amer. Math. Soc.*, **22**, 117–128 (1921).

[4] Jackson, D., *Ibid.*, 320–326 (1921).

---

*NOTE ON INVARIANCE OF DEGREE OF POLYNOMIAL AND TRIGONOMETRIC APPROXIMATION UNDER CHANGE OF INDEPENDENT VARIABLE**

BY J. L. WALSH

HARVARD UNIVERSITY

Communicated August 24, 1959

The object of this note is to show that various commonly used measures of degree of approximation are invariant under one-to-one analytic transformation of the independent variable, and this is true for both approximation by polynomials in the complex variable and trigonometric approximation in the real variable.

The methods used here have already been used several times in the literature, especially by J. H. Curtiss, Walsh and Sewell,[2] and Walsh,[5] but the present treatment is more unified and more complete, and emphasizes for the first time invariance of degrees of approximation. We call attention especially to Theorem 3 below, concerning trigonometric approximation.

The writer has recently established[6] the essence of

THEOREM 1. *Let $\epsilon_1, \epsilon_2, \ldots$ be a sequence of positive numbers approaching zero, where we suppose $\epsilon_{[n/\lambda]} = O(\epsilon_n)$ for every positive integral λ (here $[m]$ denotes the largest integer not greater than m), and where for every r, $0 < r < 1$, we have $r^n = O(\epsilon_n)$. Let E be a Jordan curve in the z-plane containing the origin in its interior, and let D be a region containing E. Let the function $f(z)$ be defined on E, let the functions $f_n(z)$ be analytic in D, and let $(n = 1, 2, \ldots)$*

$$|f(z) - f_n(z)| \leqq A_1 \epsilon_n, \qquad z \text{ on } E, \tag{1}$$

$$|f_n(z)| \leqq A_2 R^n, \qquad z \text{ in } D, \tag{2}$$

be satisfied. Then there exist polynomials $p_n(z, 1/z)$ in z and $1/z$ of respective degrees n such that

$$|f(z) - p_n(z, 1/z)| \leqq A_3 \epsilon_n, \qquad z \text{ on } E. \tag{3}$$

Here and below, the letter A with subscript denotes a positive constant independent of n and z, a constant which may change from one usage to another.

The functions $f_n(z)$ are analytic and satisfy (2) in some closed annular subregion D_1 of D bounded by two rectifiable Jordan curves J_1 to which E is interior and J_2 interior to E, with O interior to J_2. For z interior to D_1 the function $f_n(z)$ is represented as the sum of two integrals taken in the positive sense with respect to D_1:

$$f_n(z) = f_{n1}(z) + f_{n2}(z), \qquad z \text{ in } D_1, \tag{4}$$

$$f_{n1}(z) \equiv \frac{1}{2\pi i} \int_{J_1} \frac{f(t)dt}{t - z}, \qquad f_{n2}(z) \equiv \frac{1}{2\pi i} \int_{J_2} \frac{f(t)dt}{t - z}, \tag{5}$$

where $f_{n1}(z)$ is analytic throughout the interior of J_1 and $f_{n2}(z)$ is analytic throughout the exterior of J_2, even at infinity. Let $g(z)$ denote Green's function with pole at infinity for the exterior of E, and let the locus $g(z) = \log \sigma$ $(\sigma > 1)$ be generically denoted by C_σ; let $g_0(z)$ denote Green's function with pole at 0 for the interior of E, and let the locus $g_0(z) = \log \sigma$ $(\sigma > 1)$ be generically denoted by C_σ^0. From (2) and (5) follow the inequalities

$$|f_{n1}(z)| \leqq A_4 R^n, \qquad |f_{n2}(z)| \leqq A_4 R^n, \tag{6}$$

respectively on any closed set interior to J_1 and on any closed set exterior to J_2; we choose these sets to be closed Jordan regions containing E bounded respectively by suitably chosen C_ρ and C_ρ^0.

There exists [§4.5 of reference 1] for every n a sequence of polynomials $P_{nk}(z)$ in z of respective degrees $k = 1, 2, \ldots$, defined by interpolation to $f_{n1}(z)$ in points of some C_σ near E, and a sequence of polynomials $Q_{nk}(1/z)$ in $1/z$ of respective degrees

k, defined by interpolation to $f_{n2}(z)$ in points of some C_σ^0 near E, such that we have by (6)

$$\left|f_{n1}(z) - P_{nk}(z)\right| \leqq A_6 R^n/\rho_1^k, \qquad z \text{ on } E, \qquad \rho_1 > 1, \tag{7}$$
$$\left|f_{n2}(z) - Q_{nk}(1/z)\right| \leqq A_6 R^n/\rho_1^k, \qquad z \text{ on } E;$$

where A_6 is independent of k, n, and z.

From (4) and (7) we may now write

$$\left|f_n(z) - p_{n,\lambda n}(z, 1/z)\right| \leqq A_6(R/\rho_1^\lambda)^n, \qquad z \text{ on } E,$$

with $p_{nk}(z, 1/z) \equiv P_{nk}(z) + Q_{nk}(1/z)$ and with the positive integer λ so chosen that $\rho_1^\lambda > R$. By (1) and the properties of the ϵ_n we now have

$$\left|f(z) - p_{n,\lambda n}(z, 1/z)\right| \leqq A_7\, \epsilon_n. \tag{8}$$

The polynomials $p_{n,\lambda n}(z, 1/z)$ are defined only for the degrees $j = \lambda, 2\lambda, 3\lambda, \ldots$, but if we set

$$p_j(z, 1/z) \equiv p_{n,\lambda n}(z, 1/z), \ \lambda n \leqq j < \lambda\,(n+1),$$
$$p_j(z, 1/z) \equiv 0, \qquad 1 \leqq j < \lambda,$$

the polynomials $p_j(z, 1/z)$ are defined for all $j\ (\geqq 1)$, and (3) follows from (8).

A modification of Theorem 1 is of independent interest:

COROLLARY. *If in Theorem 1 the functions $f_n(z)$ are analytic also throughout the interior of E (that is, if D contains E and its interior), then the polynomials $p_n(z, 1/z)$ in (3) may be chosen as polynomials in z.*

In the proof we set $f_{n2}(z) \equiv 0$, $Q_{nk}(1/z) \equiv 0$.

It will be noticed that the hypothesis of Theorem 1 is invariant under suitable one-to-one conformal transformation of the region D. Moreover, if the $f_n(z)$ satisfying (1) are given as polynomials of degree n in z and $1/z$, then [Lemma, p. 259 of reference 1] (2) is a consequence of (1). There follows

THEOREM 2. *Let the ϵ_n, E, D, and $f(z)$ satisfy the conditions of Theorem 1. If $f(z)$ can be approximated by polynomials in z and $1/z$ of respective degrees n on E with degree of approximation $O(\epsilon_n)$, if D is mapped one-to-one and conformally onto a region D_1 of the w-plane with E transformed into E_1 containing $w = 0$ in its interior and if $f(z)$ is transformed into $f_1(w)$, then $f_1(w)$ can be approximated by polynomials in w and $1/w$ of respective degrees n on E_1 with degree of approximation $O(\epsilon_n)$. Thus the class of functions $f_1(w)$ which can be approximated by polynomials in w and $1/w$ on E_1 with degree of approximation $O(\epsilon_n)$ is identical with the class of the transforms of functions $f(z)$ which can be approximated by polynomials in z and $1/z$ on E with degree of approximation $O(\epsilon_n)$.*

In particular, if D contains both E and the interior of E, only polynomials in z and in w respectively may be involved.

If E and E_1 are arbitrary *analytic* Jordan curves, there exist regions D and D_1 and an infinity of transformations of the kind required, even if D contains both E and its interior. Either E or E_1 may be chosen as the unit circle:

COROLLARY 1. *Let E be an analytic Jordan curve of the z-plane containing $z = 0$ in its interior, let the ϵ_n satisfy the conditions of Theorem 1, and let the image of E under a one-to-one conformal map be E_1: $|w| = 1$. The class of functions $f(z)$ that can be*

approximated on E by polynomials in z and $1/z$ of respective degrees n with degree of approximation $O(\epsilon_n)$ is identical with the class of the transforms of functions $f_1(w)$ that can be approximated on E_1 by polynomials in w and $1/w$ of respective degrees n with degree of approximation $O(\epsilon_n)$.

In particular, if the map transforms the interior of E onto the interior of E_1 one-to-one and conformally, only polynomials in z and in w respectively may be involved.

Choice of E in Corollary 1 as $|z| = 1$ gives

COROLLARY 2. *Under the conditions of the first or second part of Corollary 1, the class of functions $f(z)$ involved is invariant under a one-to-one conformal transformation which maps E_1 onto itself.*

It is readily shown [compare Lemma 2 of reference 4] that a one-to-one analytic map with nonvanishing derivative of an analytic Jordan curve onto another is one-to-one and analytic in suitable regions containing those curves in their respective interiors.

Corollaries 1 and 2 are of especial significance in that they refer to the unit circle E_1: $|w| = 1$, on which any polynomial in w and $1/w$ of degree n is also a trigonometric polynomial in $\theta = \arg w$ of order n, and conversely, by virtue of the Euler relations $w^k = e^{ik\theta} = \cos k\theta + i \sin k\theta$. Moreover approximation on E_1 to a real function by polynomials in w and $1/w$ of degree n is equivalent to approximation on E_1 by real trigonometric polynomials of order n, with the same degree of approximation. Thus we have

THEOREM 3. *If ϵ_n satisfies the conditions of Theorem 1, the class of real functions of period 2π that can be approximated by trigonometric polynomials of respective orders n with degree of approximation $O(\epsilon_n)$ is invariant under one-to-one analytic map $x' = \phi(x)$ of the axis of reals onto itself with nonvanishing derivative and with $\phi(x + 2\pi) \equiv \phi(x) + 2\pi$.*

Further, if this map is of power series type when interpreted on the unit circumference, the class of functions of period 2π that can be approximated by trigonometric polynomials of respective orders n of power series type with degree of approximation $O(\epsilon_n)$ is invariant.

If in Theorem 3 we have $\epsilon_n = 1/n^{p+\alpha}$, where p is a nonnegative integer and $0 < \alpha < 1$, the class of functions $f(x)$ involved is the class for which $f^{(p)}(x)$ exists and satisfies a Lipschitz condition in x of order α, as is known by the Bernstein-Jackson-Montel-de la Vallée Poussin theory; for this class the invariance is easy to prove. But with such a choice as $\epsilon_n = 1/n \log n$ $(n \geqq 2)$, the invariance is by no means obvious. Zygmund has introduced special classes of functions characterized by $\epsilon_n = 1/n^{k+1}$ in Theorem 3, namely the class of functions possessing continuous derivatives of the kth order satisfying a uniform condition

$$|F(x + h) + F(x - h) - 2F(x)| \leqq A|h|;$$

the invariance also of these classes follows from Theorem 3. The analogous classes on an arbitrary analytic Jordan curve were introduced and studied by Walsh and Elliott.[3]

Theorem 1 refers to the Tchebycheff measure of approximation both in hypothesis (1) and conclusion (3). However, the conclusion persists if (1) is replaced by the inequality

$$\int_E |f(z) - f_n(z)|^p |dz| \leqq A_1 \epsilon_n{}^p, \qquad p > 0, \tag{9}$$

and (3) replaced by the corresponding inequality

$$\int_E |f(z) - p_n(z, 1/z)|^p |dz| \leqq A_3 \epsilon_n^p, \tag{10}$$

where of course we assume E rectifiable. In the proof, no essential change needs to be made, although we use the standard inequalities

$$|\chi_1 + \chi_2|^p \leqq 2^{p-1}[|\chi_1|^p + |\chi_2|^p], \qquad p > 1,$$
$$|\chi_1 + \chi_2|^p \leqq |\chi_1|^p + |\chi_2|^p, \qquad 0 < p \leqq 1;$$

the analog of the Corollary persists.

Likewise Theorem 2, Corollary 1, Corollary 2, and Theorem 3 have valid analogs involving integral measures of approximation as in (9) and (10). In the proof we need to derive (2) as a consequence of such an inequality as (10); here the above standard inequalities yield

$$\int_E |p_n(z, 1/z)|^p |dz| \leqq A_0, \tag{11}$$

whence [Lemma of reference 2] we have

$$|p_n(z, 1/z)| \leqq A_1 \rho^n, \qquad \rho > 1, \tag{12}$$

for z in the annular region bounded by the curves C_ρ and C_ρ^0 used in the proof of Theorem 1.

Hardy and Littlewood have introduced classes of functions whose kth derivatives satisfy integrated pth power ($p \geqq 1$) Lipschitz conditions of order α, $0 < \alpha < 1$; if E is analytic these classes are characterized by (10) with $\epsilon_n = 1/n^{k+\alpha}$. Proofs in the trigonometric case were provided by Quade, in the case of analytic functions (where the integrated Lipschitz conditions are taken with respect to arc length) by Walsh and Russell.[4] The proof of (10) from (9) establishes anew[4] the invariance of these classes under suitable conformal transformation. There are corresponding results for trigonometric and polynomial approximation to functions whose kth derivatives satisfy an integrated pth power Zygmund condition, in the case of polynomial approximation with respect to arc length; the class of functions is likewise invariant.

If the Corollary to Theorem 1 is modified so that (1) and (3) are replaced not by inequalities involving line integrals as in (9) and (10) but surface integrals:

$$\iint_{E^*} |f(z) - f_n(z)|^p \, dS \leqq A_1 \epsilon_n^p, \qquad p > 0, \tag{13}$$

$$\iint_{E^*} |f(z) - p_n(z)|^p \, dS \leqq A_2 \epsilon_n^p, \tag{14}$$

taken over the interior E^* of E, the conclusion persists. Likewise the analogs of the latter part of Corollary 1 to Theorem 2 and of the latter part of Theorem 3 are valid. In the modified proof we derive

$$\iint_{E^*} |p_n(z)|^p \, dS \leqq A_4 \tag{15}$$

as a consequence of (14), whence [§5.3, Lemma 2; §2.1, Theorem 2; §4.60 Lemma, all of reference 1] we have

$$|p_n(z)| \leqq A_3\sigma_1^n, \qquad 1 < \rho < \sigma_1, \tag{16}$$

throughout the interior of the curve C_ρ used in the proof of Theorem 1.

In the important case $p = 2$, E: $|z| = 1$, the sum of the first $n + 1$ terms of the Maclaurin development of $f(z)$, with the hypothesis of the second part of Corollary 1 to Theorem 2, is the polynomial $p_n(z)$ of degree n of best approximation to $f(z)$ on both E and E^*. There is then a close relation[6] between the two measures of best approximation, say the first members M_n^2 and M_n^{*2} of (10) and (14), namely

$$M_n^{*2} \leqq M_n^2/2\ (n + 2). \tag{17}$$

If μ_n^2 and μ_n^{*2} denote the corresponding measures of approximation after conformal map of E^* onto the interior of an analytic Jordan curve, we have (if M_n and M_n^* satisfy the conditions of Theorem 1 on ϵ_n)

$$\mu_n^{*2} \leqq A_1 M_n^{*2} \leqq A_1 M_n^2/2\ (n + 2) \leqq A_2\mu_n^2/2\ (n + 2). \tag{18}$$

Thus (17) is invariant under conformal transformation, subject to an additional constant factor on the right.

The derivation of (17) and (18) also applies to approximation on E: $|z| = 1$ and E^* by harmonic polynomials, which on E is equivalent to approximation by trigonometric polynomials.

Throughout this paper, positive continuous weight functions can be inserted in such inequalities as (1), (3), (9), (10), (13), (14), (17), (18) without altering the truth of our conclusions.

DEDICATED TO MARSTON MORSE as one of a series of articles so dedicated by a number of associates cooperating to honor his mathematical achievements.

* This research was sponsored (in part) by Contract AF 18(600)-1461 with the U. S. Air Force, Air Research and Development Command.

[1] Walsh, J. L., *Interpolation and Approximation*. (New York: 1935). Vol. 20, Colloquium Publications of the American Mathematical Society.

[2] Walsh, J. L., and W. E. Sewell, *Bull. Amer. Math. Soc.*, **46**, 312–319 (1940).

[3] Walsh, J. L., and H. M. Elliott, *Trans. Amer. Math. Soc.*, **68**, 183–203 (1950).

[4] Walsh, J. L., and H. G. Russell, *Trans. Amer. Math. Soc.*, **92**, 355-370 (1959).

[5] Walsh, J. L., *Math. Zeit.* (forthcoming).

[6] Walsh, J. L., *Proc. Amer. Math. Soc.*, **10**, 273–279 (1959).

---

*BIOASSAY OF ORGANIC MICRONUTRIENTS IN THE SEA**

BY WILLIAM L. BELSER

SCRIPPS INSTITUTION OF OCEANOGRAPHY

Communicated by Carl L. Hubbs, August 14, 1959

Introduction.—It has often been stated[1] that oceanic production is limited by the quantity of phosphate in the marine environment. The best data available indicate that the concentration of phosphate in the water off the coast of California is about ten times as high as that found in the English Channel. Yet the total productivity

of the English Channel area has been consistently greater than that found off our coast. Why is it that even in the presence of sufficient concentrations of the essential inorganic nutrients, our Pacific coast is barren relative to that of an area poor in those same inorganic substances? Although it is possible that variability in the phosphorus-nitrogen ratio might in part account for this lower production, it would seem more reasonable to explore the situation further in terms of other limiting substances. As a result of a number of independent observations over a period of years, attention has been focused on organic, rather than on inorganic substances as limiting agents. The literature on the subject has been reviewed by Lucas,[2] Provasoli,[3] and Margalef.[4] I shall cite only a few of the pertinent papers, dealing with the implication of organic micronutrients in marine biology. Historically, Allen[5] in 1914 observed that in the absence of either natural sea water or an extract of *Ulva* (a marine alga), the diatom *Thalassorsira gravida* failed to grow in his artificial sea water. In some more recent experiments with algae, Harvey[6] has found that *Ditylium brightwelli*, another diatom, cannot be grown continuously even in natural sea water enriched with nitrate, phosphate, and iron. He suggested that accessory substances, probably of organic nature, are required for continuous production. As more and more marine algae are being cultured in the laboratory, in defined media and in the absence of bacteria, some of the organic compounds required for their growth are being uncovered. Lewin[7] has demonstrated an absolute requirement for cobalamin by a marine *Stichococcus*, and Sweeney[8] has found that the same vitamin is necessary for the growth of *Gymnodinium*. Droop[9] has found requirements for both vitamins B_{12} and B_6 in a variety of algae. Galactose, a monosaccharide, has been shown by Collier[10] to stimulate filtering rate in oysters. Loomis[11] reported that the feeding response in Hydra is triggered by glutathione. Wilson[12] has postulated that the basis for the discontinuous distribution of bottom communities of invertebrates may reside in some minute quantities of organic material, since exhaustive analysis of physical and inorganic chemical conditions fails to yield the answers to why the organisms aggregate. The red tide, a profuse growth of marine algae, has been shown to be markedly stimulated by vitamin B_{12}. The occurrence of red tides has been correlated with the presence of vitamin B_{12} in near-shore waters and also with reduced salinities resulting, from rain run-off from the land. Even though not absolutely required, certain organic substances have been found to stimulate growth. Lewin,[13] for example, has demonstrated that a marine alga, *Prasiola stipitata*, can grow in the absence of organic material, but is markedly stimulated by the presence of organic nitrogen.

Wilson's observations[12] on the distribution of marine invertebrates certainly suggest that some soluble or small particulate organic substance may be involved, and such phenomena as plankton succession and initiation of plankton blooms may be explainable on the basis of growth factors, antibiotics, and other stimulatory and inhibitory compounds. Many attempts have been made to correlate the patchy distribution of plants and animals, bloom initiation, and bloom succession with inorganic chemicals, light and salinity, but the data are far from conclusive. Variations in the inorganic consitutents do not seem adequate to cause the marked variations in the biological phenomena involved. For these reasons, it seemed reasonable to attempt to establish a method that would detect small amounts of

organic materials in sea water and would possibly indicate a basis for these dynamic processes.

A number of attempts have been made to isolate and characterize organic materials from sea water by biochemical techniques, but most of these attempts have been cumbersome and time-consuming. Moderate success was encountered by Provasoli and Pintner,[14] and Johnston,[15] in extracting and concentrating organic material, but it seems likely that a biological assay would be more sensitive than even the most refined biochemical techniques, and might detect many substances that would be missed entirely by those techniques. One of the most sensitive and useful techniques in biochemistry, chromatography, is limited in its application, since its use requires that the water first be desalted, or that the organic substances be adsorbed on some agent, then eluted and concentrated prior to chromatography. The high salt concentrations in sea water preclude direct chromatography.

The response of biochemically deficient mutant strains of bacteria to their specific metabolites suggested that it might be possible to utilize these mutants for a qualitative, and perhaps even quantitative, bioassay. They respond to very low concentrations of biochemicals and are relatively specific in their response. Attempts to train *Escherichia coli*, in which many mutants are already in culture, to grow in sea water were impractical. After as many as fifteen transfers, only sporadic growth was obtained. Therefore, a number of marine bacteria were screened for desirable characteristics, and *Serratia marinorubra* was selected as the most suitable organism.

Materials and Methods.—*Serratia marinorubra*[16] is a gram-negative coccobacillus, 0.3 by 0.6 microns in size, and is characterized by its red pigment. It grows well in a medium composed of inorganic salts with glycerol as the sole source of energy. The organism has a broad salt tolerance: it grows in both fresh water and three-times concentrated sea water. Stock cultures are maintained on slopes containing 1.5 per cent agar, 2.0 per cent glycerol, and 0.2 per cent peptone in sea water. This nutritionally independent or "wild" type stock is labeled SIO.

Mutant cultures with requirements for biochemical compounds have been induced by ultraviolet light, and isolated by a variety of techniques. The organisms may be grown either in liquid medium or on agar surfaces prior to irradiation. If grown on agar surfaces, the organisms are picked with a wire loop and suspended in sterile water for the irradiation. Those grown in liquid medium are washed several times by centrifugation-decantation, and the final pellet suspended in sterile water. The suspension resulting from either of these methods is then visually adjusted to approximately 10^8 organisms per ml turbidity and 7 ml is delivered into a sterile petri plate. This volume gives a relatively uniform 1-mm-thick layer in the Petri plate. The plate is placed on a ring-stand at a distance of 50 cm from the ultraviolet source and is treated for various times. During irradiation, the organisms are agitated in the suspension by a small stirring motor, which is attached to the ring-stand and which has its shaft deliberately thrown out of center. This imparts a vibrational motion to the ring-stand, and very effectively agitates the bacterial suspension, thus ensuring that the bacteria receive a random and relatively uniform dose of irradiation per cell. We have found best production of mutants in the range of 0.01 to 0.001 per cent survival, and this is obtained by exposing the suspension for periods of 60 to 80 seconds. The approximate dose of ultraviolet

delivered in 60 seconds is 1000 ergs/mm.^{2} After irradiation, several methods are routinely used to recover the mutants. The treated suspension may be plated on agar medium containing complete enrichments, so that all mutant and nutritionally independent cells will rapidly attain a profuse colonial growth. By taking advantage of the Lederberg and Lederberg[17] replica plating technique, these colonies can be transferred to a sterile velvet pad and in turn from that imprinted on minimal and complete media. Those colonies which grow on complete but not minimal medium have lost the ability to synthesize some biochemical necessary for their growth and are transferred with a wire loop to an agar slope for later analysis. Another method for recovery of mutants from the irradiated suspension is to plate the treated cells on agar medium which contains a limiting concentration of enrichment. The nutritionally independent cells surviving the irradiation will grow and produce normal-sized colonies, while any biochemically deficient mutants will grow only until they have exhausted the local environment of the small concentration of their specific substrate which has been added, at which point growth will be arrested. By taking advantage of the resulting difference in colony size, the small colonies can be picked with a wire loop and tested by inoculating complete and minimal medium with the suspected mutant, as above.

When they have been isolated and are in culture on agar-slope tubes, the mutant stocks may be tested for their specific requirements. The commonly recognized biochemical constituents of most microbial media have been made up into pools for convenience in testing. These pools are as follows:

Pool 1. Essential amino acids.
Pool 2. Non-essential amino acids.
Pool 3. Purines and pyrimidines (bases, ribosides, and ribotides).
Pool 4. Vitamins (a total of 22 vitamins in 50 per cent ethanol).

Agar plates are prepared which contain complete medium, minimal medium, and minimal medium containing the above pools, one in each plate. This gives a total of six plates, and the mutant is streaked with a wire loop on the surface of each of these six plates. The complete medium plate is a viability control, and the mutant grows thereon. The minimal-medium control is used for a reverse mutation check, for if the mutant is stable, no growth should be observed. If the mutant grows on one of the pools, then it is a simple matter to check the individual compounds comprising that pool for their ability to satisfy the requirement of the mutant. For the purpose of our bioassay system, mutants that do not respond to one of the pools are not tested further but are held separate for other work. Mutants that do respond to a pool are further tested as follows: A heavy suspension of the mutant is spread uniformly over the surface of a minimal-medium plate. By definition, the mutant will not grow on this plate in the absence of added biochemicals. Now crystals of each of the biochemical compounds comprising the pool are dropped on to the surface of the agar, and the plates are incubated. After 24 to 48 hours incubation, the specific component of the pool that satisfies the requirement of the mutant will be surrounded by a marked zone of growth, the size of which will depend on the rate of diffusion through the agar. The mutant is then labeled appropriately and its quantitative response to graded concentrations of its substrate is determined.

The minimal medium generally used in these experiments is that devised by Labrum and Bunting[18] for *Serratia marcescens*. For complete medium, this minimal medium is supplemented with yeast extract, casitone, and Bacto-peptone (2 gm of each per liter of minimal medium).

MINIMAL MEDIUM

K_2HPO_4 (anhydrous)	8.0 gm	Glycerol	20.0 ml
$(NH_4)_2HC_6H_5O_7$	5.0 gm	Distilled water	1.0 *l*
$MgSO_4 \cdot 7H_2O$	0.5 gm	Bacto-agar (optional)	15.0 gm
$FeNO_3 \cdot 9H_2O$	0.02 gm		

The application of these mutants to tests of sea water, including sampling methods, sterilization, and other procedures will be discussed in the experimental results section, along with the pertinent data.

Experimental Results.—By application of the techniques described in the preceding section, a number of nutritionally exacting mutant strains have been isolated. A list of the requirements of the mutants obtained to date follows:

Uracil	(3)	Histidine (Proline)	(2)
Tryptophane	(1)	Methionine (Cystine)	(1)
Threonine	(1)	Glycine	(1)
Isoleucine	(1)	Purine*	(20)—One culture lost in transfer
Biotin	(1)—Lost in transfer	Peptone†	(3)
Adenine	(2)	Arginine	(1)

The number in parentheses following the biochemical indicates the number of separate mutants isolated with that requirement. The asterisk following purine indicates a nonspecific requirement for those mutants. All mutants respond to all of the purine bases, their ribosides and their ribotides. The dagger following peptone indicates that these three mutants respond to some component of peptone, probably complex, which is as yet unidentified.

At various times during the course of the isolation of the biochemical mutants, samples of sea water have been subjected to test analysis, using the mutants then available. Each group of samples will be considered separately in the following section, since different numbers of mutants were used.

The first group of water samples subjected to bioassay with mutants of *Serratia marinorubra* were collected for me by Dr. Galen E. Jones on a cruise in waters off Costa Rica.[19] The samples were taken in two ways. Surface samples were obtained by collecting with a clean plastic bucket, lowered over the side of the research vessel as soon as she was on station. Samples from various depths were taken in vertical casts using the Van Dorn sampler. All samples were immediately passed through Whatman No. 2 filter paper to remove large particulate material and then delivered into screw cap bottles and autoclaved. The samples were brought back to the laboratory for tests and were stored for varying times before testing. When the samples were returned to the laboratory, tests were set up as follows:

Sterile glycerol was added to the bottle to serve as energy source and two ml aliquots of each sample were delivered into sterile tubes. All tests were run with

triplicate tubes. One set of tubes from each sample was held without inoculation as sterility controls, and one set was inoculated with each mutant. Another set was inoculated with the nutritionally independent parent culture as a viability control to make sure that the sea water could support growth of the bacteria and that no antibiotic substances were present. For each group of samples tested, a reversion control was run on each mutant to insure that reverse mutation to nutritional independence did not give false positive tests. Following a suitable period

TABLE 1

PRESENCE OF GROWTH FACTORS IN SEA-WATER SAMPLES AS INDICATED BY BACTERIAL MUTANT GROWTH AND CORRELATIONS WITH CHLOROPHYLL DETERMINATIONS AND NUMBERS OF FREE BACTERIA IN THE SAME LOCATIONS*

Date of Sample	SC	B	U	P	Depth in Meters	Chlorophyll, mg/m³†	Depth	Bacterial Count/ml‡
11/10/56	0	0	0	0	Surface	0.125	Surface	2
11/12/56	0	0	0	0	Surface	0.204	Surface	No count
11/15/56	0	++	0	0	Surface	0.577	Surface	34
11/16/56	0	0	0	0	Surface	0.380	Surface	546
11/21/56	0	±	0	0	Surface	0.308	Surface	...
"	0	±	0	0	5	...	...	...
"	0	0	0	0	10	0.310	8	...
"	0	±	0	0	14	0.340	12	...
"	0	0	0	0	18	0.342	16	187§
"	0	±	±	0	22	0.335	20	4,200§
"	0	0	0	0	27	0.458	30	383§
"	0	0	0	0	50	...	...	...
"	0	0	0	0	100	0.280	100	3,200§
"	0	0	0	0	200	0.092	200	...
12/1/56	0	0	0	0	Surface	0.329	Surface	75
"	0	0	±	++	10	0.364	10	3
"	0	0	±	0	50	0.491	50	1
"	0	++	0	0	75	0.101	100	1
12/2/56	0	0	0	0	Surface	0.196	Surface	23
"	0	++	0	0	5	No reading	5	6
"	0	++	0	0	10	0.215	10	5
"	0	++	+	0	25	0.261	25	2
"	0	++	+	0	50	0.633	50	15
"	0	+	0	0	75	No reading	75	5
"	0	±	0	0	100	0.105	100	2
12/3/56	0	+	0	0	Surface	0.169	Surface	114
12/5/56	0	++	0	0	Surface	0.124	Surface	97
12/7/56	0	++	0	0	Surface	No reading	Surface	7
"	0	++	0	0	25	0.905	25	14

* Each water sample was enriched with glycerol and equal volumes were delivered into four tubes. One was not inoculated, and served as a sterility control (SC). The other three, respectively, were inoculated with mutants requiring biotin (B), uracil (U), and purine (P). The tubes were scored after five days according to the following visual turbidity index: 0 = no turbidity, ± = faint turbidity, + = slight but definite turbidity, ++ = moderate and +++ = heavy turbidity.

§ These figures not considered valid because of type of sampler used.

† Data of R. W. Holmes.

‡ Data of Galen E. Jones.

of incubation at ambient temperatures, the turbidity in the tubes was visually estimated. It is possible to read these tests in the Beckman DU spectrophotometer for quantitative estimation, but at this stage it seems more important to document the occurrence and distribution of nutrients than to attempt to quantitate them.

When these samples were taken, only three mutants were available for the test. The results of these tests appear in Table 1.

These data indicate that soluble organic micronutrients are present in the sea

and that they are sporadically distributed both horizontally and vertically. The results indicated that the technique has merit for this sort of work and encouraged us to continue isolating mutants for the assay. No attempts were made to test sea-water samples further until a sufficiently broad spectrum of nutritional mutant strains were available.

By November, 1958, we had isolated ten different mutants and had identified their requirements. These seemed to represent a sufficiently broad spectrum of assayable micronutrients to justify a rigid trial at sea. Arrangements were made for a technician to accompany a cruise to the Gulf of Tehuantepec under the auspices of the Scripps Tuna Oceanography Research Program of the U. S. Bureau of Commercial Fisheries. Samples were secured at the entrance to the Gulf, and at a number of stations in the Gulf. All of the samples taken on the Tehuantepec cruise were obtained by using the Van Dorn plastic sampler. A total of seven surface samples and nine 4-bottle vertical casts were taken. As soon as they were brought aboard, the samples were passed through Whatman No. 2 filter paper. About 150 ml of the filtrate from each sample were immediately delivered into screw-cap bottles, autoclaved, and stored at room temperature for future testing in the laboratory. Glycerol was added to the remaining portion of the sample, which was sterilized by one of two methods. Part of the experimental protocol was designed to test differences between autoclaving and sterile filtration, and the samples were either Seitz filtered and then added to sterile tubes, or added directly to tubes and then sterilized by autoclaving. These tubes were inoculated with the various mutant strains and incubated. They were scored at 4 and 8 days for turbidity. Some samples were incubated at ambient temperatures, others in a 29°C incubator, and still others in a water bath with circulating sea water at ambient temperatures.

All samples proved negative for organic materials by our test, and in fact, the nonexacting parental strain also failed to grow. Spot checks for antibiotic activity were also negative. It was suspected that possibly some inorganic constituent of the medium might be limiting in view of the failure of the nonexacting strain to grow, and so several tubes were supplemented with our basal medium. After this addition, the parental stock did grow and turbidity developed in those tubes. It was not until the samples were checked further in the laboratory that the limiting inorganic chemical was identified as the nitrogen source. As a result of a rather extensive set of experiments on the inorganic chemicals, the technique has been modified, and inorganic enrichment has been made a part of our protocol. The failure to get positive tests for organic micronutrients on this cruise, however, does not detract from the value of the method. The results confirmed the absolute nature of the response of the mutants to their specific substrates, and the application of the technique at sea for a period of five weeks permitted revision and improvement of the shipboard protocol.

The most extensive application of the bioassay method to sea-water samples has been in progress since December 15, 1958. On that date, there was initiated a series of twice-weekly samples from the Scripps Pier at La Jolla, California. The purpose of these samples is to examine changes over a period of time at one station, to give the technique a stringent test in terms of reproducibility, and to attempt to integrate the program with other types of work being done on the same samples.

As a result of the data from the Tehuantepec samples, we have included inorganic enrichment in all of these tests. The modified protocol for the pier samples is as follows:

Surface samples are taken by a bucket cast. The sample is filtered through Whatman No. 2 paper. Glycerol is added to the sample, and two ml aliquots are delivered into one set of tubes. Phosphate and nitrate enrichment is added to the remainder of the sample, and another set of tubes is prepared by sterile filtration. (This latter set is used in the tryptophane assay, since tryptophane breaks down on autoclaving.) The remainder of the enriched sample is delivered into tubes, and all but the tryptophane set are autoclaved. All tubes are inoculated with the mutant organisms, and one set is held as a sterility control. The nonexacting parental strain is always included as a viability control to detect antibiotic activity or inorganic chemical anomalies. In this series of tests, the biochemical requirements of the mutants are as follows:

Uracil	Peptone
Isoleucine	Threonine
Methionine	Tryptophane
Histidine	Purine
Adenine	Glycine

The results of these samples are best presented in graph form, since only two of them have occurred with regularity. A graph of the fluctuations in isoleucine and uracil appears in Figure 1. Glycine appeared in two samples, and all samples were completely negative for the other seven mutants.

General Discussion.—Although the data that we have obtained thus far in the program are highly provocative, it is felt that much more work is needed before we can attempt very definite conclusions in regard to the specific problems outlined in the introduction. The data do, however, constitute an adequate test of the bioassay technique, from the qualitative standpoint. The method is reliable, is reproducible, and can be controlled for reverse mutation that might give false results.

The discontinuity in the distribution of the biochemical compounds found in Table 1 both vertically and horizontally in the ocean is quite interesting. One would expect *a priori*, that these would exhibit a gradient effect in an aqueous environment, showing high concentration in a center, and diminishing concentrations toward the periphery. The observed discontinuity may be a reflection of localized physical phenomena such as internal waves, shearing effects of currents, and other factors of this nature which would impinge on a normal diffusion gradient. This aspect of the problem is amenable to test, and must be examined before definite conclusions are possible.

In the most recent phase of the work, where the bioassay has been applied to samples obtained from the Scripps Pier, there is an obvious variation in the concentration of biochemicals at a given point over a period of time. At this stage in the work, it is impossible to explain these fluctuations on the basis of the scant data available. The differences in concentration are real, but the underlying cause is unknown. It is interesting to speculate that the differences may result from a flow of water past the pier, or from biological uptake, but only with more data and refined techniques will definite interpretation be possible.

Summary.—A bioassay technique for organic micronutrients in sea water is described. The use of biochemically deficient mutant strains of a marine bacterium for this purpose is a novel approach, and the preliminary data indicate that the assays are valid.

The results of the application of the bioassay indicate that sea-water does, indeed, contain organic micronutrients. Of the ten substances assayed for, biotin, uracil, and isoleucine appeared most frequently. One occurrence of purine and two isolated occurrences of glycine were noted. More recently, tryptophan and threonine have each appeared in one sample.

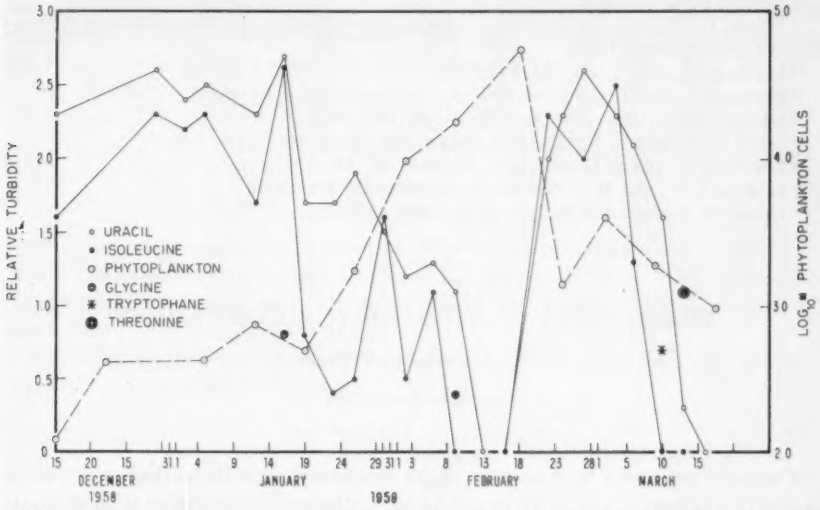


FIG. 1.—Occurrence of organic micronutrients in samples taken from Scripps Pier. Although ten different biochemicals were assayed for, only five were detected during this time period. The curve showing phytoplankton numbers is from data of Dr. B. M. Sweeney and Anne N. Dodson.

A series of weekly samples from a shore station have given much valuable information concerning the validity and reproducibility of the technique.

It is expected that extended application of the bioassay technique will elucidate some unexplained discontinuities in the distribution of marine organisms and may be of value in the description and identification of water masses and in tracing their movements.

The criticisms and suggestions made by Dr. Galen E. Jones on the experimental work have been particularly helpful, and the technical assistance of Miss Phyllis Bear and Mr. William A. Moller is gratefully acknowledged. The author would like to express his gratitude to Dr. Sweeney and Mrs. Dodson for making their data on phytoplankton populations available. He would also like to thank Dr. Carl L. Hubbs for his critical review on the manuscript.

* Contribution from the Scripps Institution of Oceanography, New Series. This work was initiated during the tenure of a U. S. Public Health Service Postdoctoral fellowship from the National Cancer Institute (CF-5737G), and has been supported by grants from the National Science Foundation (#G-4402) and from the Nutrilite Corporation.

[1] Redfield, A. C., *Am. Scientist*, **46**, 205 (1958).
[2] Lucas, C. B., *Biol. Rev.*, **22**, 270 (1947).
[3] Provasoli, L., in Buzzati-Traverso, *A Symposium on Perspectives in Marine Biology* (University of California Press, 1958).
[4] Margalef, R., *Ibid.*
[5] Allen, E. J., *J. Mar. Biol. Assn. U. K.*, **10**, 417 (1914).
[6] Harvey, H. W., *Ibid.*, **23**, 499 (1939).
[7] Lewin, R. A., *J. Gen. Microbiol.*, **10**, 93 (1954).
[8] Sweeney, B. M., *Am. J. Bot.*, **41**, 821 (1954).
[9] Droop, M. R., *J. Mar. Biol. Assn. U. K.*, **37**, 323 (1958).
[10] Collier, A., *et al.*, *Science*, **111**, 151 (1950).
[11] Loomis, F., unpublished, 1953.
[12] Wilson, D. P., in Buzzati-Traverso, *A Symposium on Perspectives in Marine Biology* (University of California Press, 1958).
[13] Lewin, R. A., *Can. J. Bot.*, **33**, 5 (1955).
[14] Provasoli, L., and I. J. Pintner, *Proc. Soc. Protozoologists*, **4**, 10 (1953).
[15] Johnston, R., *J. Mar. Biol. Assn. U. K.*, **34**, 185 (1955).
[16] Zobell, C. E., and H. Upham, *Bull. Scripps Inst. Oc.*, **5**, 239 (1944).
[17] Lederberg, J., and E. Lederberg, *J. Bacteriol.*, **63**, 399 (1952).
[18] Labrum, E. L., and M. I. Bunting, *J. Bacteriol.*, **65**, 394 (1953).
[19] Belser, W. L., *Spec. Sci. Repts.-Fisheries #279*, 55 (1958).

THE ALGEBRA OF MICROSCOPIC MEASUREMENT

BY JULIAN SCHWINGER

HARVARD UNIVERSITY

Communicated August 28, 1959

This note initiates a brief account of the fundamental mathematical structure of quantum mechanics, not as an independent mathematical discipline with physical applications, but evolved naturally as the symbolic expression of the physical laws that govern the microscopic realm.[1]

The classical theory of measurement is implicitly based upon the concept of an interaction between the system of interest and the measurement apparatus that can be made arbitrarily small, or at least precisely compensated, so that one can speak meaningfully of an idealized measurement that disturbs no property of the system. The classical representation of physical quantities by numbers is the identification of all properties with the results of such nondisturbing measurements. It is characteristic of atomic phenomena, however, that the interaction between system and instrument cannot be indefinitely weakened. Nor can the disturbance produced by the interaction be compensated since it is only statistically predictable. Accordingly, a measurement of one property can produce uncontrollable changes in the value previously assigned to another property, and it is without meaning to ascribe numerical values to all the attributes of a microscopic system. The mathematical language that is appropriate to the atomic domain is found in the symbolic transcription of the laws of microscopic measurement.

The basic concepts are developed most simply in the context of idealized physical systems which are such that any physical quantity A assumes only a finite number of distinct values, $a', \ldots a^n$. In the most elementary type of measurement, an

ensemble of independent similar systems is sorted by the apparatus into subensembles, distinguished by definite values of the physical quantity being measured. Let $M(a')$ symbolize the selective measurement that accepts systems possessing the value a' of property A and rejects all others. We define the addition of such symbols to signify less specific selective measurements that produce a subensemble associated with any of the values in the summation, none of these being distinguished by the measurement. The multiplication of the measurement symbols represents the successive performance of measurements (read from right to left). It follows from the physical meaning of these operations that addition is commutative and associative, while multiplication is associative. With 1 and 0 symbolizing the measurements that, respectively, accept and reject all systems, the properties of the elementary selective measurements are expressed by

$$M(a')M(a') = M(a') \tag{1}$$

$$M(a')M(a'') = 0, \qquad a' \neq a'' \tag{2}$$

$$\sum_{a'} M(a') = 1. \tag{3}$$

Indeed, the measurement symbolized by $M(a')$ accepts every system produced by $M(a')$ and rejects every system produced by $M(a'')$, $a'' \neq a'$, while a selective measurement that does not distinguish any of the possible values of a' is the measurement that accepts all systems.

According to the significance of the measurements denoted as 1 and 0, these symbols have the algebraic properties

$$1\,1 = 1, \qquad 0\,0 = 0$$
$$1\,0 = 0\,1 = 0$$
$$1 + 0 = 1,$$

and

$$1M(a') = M(a')1 = M(a'), \; 0M(a') = M(a')0 = 0$$
$$M(a') + 0 = M(a'),$$

which justifies the notation. The various properties of 0, $M(a')$, and 1 are consistent, provided multiplication is distributive. Thus,

$$\sum_{a''} M(a')M(a'') = M(a') = M(a')1 = M(a')\sum_{a''} M(a'').$$

The introduction of the numbers 1 and 0 as multipliers, with evident definitions, permits the multiplication laws of measurement symbols to be combined in the single statement

$$M(a')M(a'') = \delta(a',a'')M(a'),$$

where

$$\delta(a',a'') = \begin{cases} 1, & a' = a'' \\ 0, & a' \neq a''. \end{cases}$$

Two physical quantities A_1 and A_2 are said to be compatible when the measurement of one does not destroy the knowledge gained by prior measurement of the

other. The selective measurements $M(a_1')$ and $M(a_2')$, performed in either order, produce an ensemble of systems for which one can simultaneously assign the values a_1' to A_1 and a_2' to A_2. The symbol of this compound measurement is

$$M(a_1'a_2') = M(a_1')M(a_2') = M(a_2')M(a_1').$$

By a complete set of compatible physical quantities, $A_1, \ldots A_k$, we mean that every pair of these quantities is compatible and that no other quantities exist, apart from functions of the set A, that are compatible with every member of this set. The measurement symbol

$$M(a') = \prod_{i=1}^{k} M(a_i')$$

then describes a complete measurement, which is such that the systems chosen possess definite values for the maximum number of attributes; any attempt to determine the value of still another independent physical quantity will produce uncontrollable changes in one or more of the previously assigned values. Thus the optimum state of knowledge concerning a given system is realized by subjecting it to a complete selective measurement. The systems admitted by the complete measurement $M(a')$ are said to be in the state a'. The symbolic properties of complete measurements are also given by equations (1), (2), and (3).

A more general type of measurement incorporates a disturbance that produces a change of state. (It is here that we go beyond previous developments along these lines.) The symbol $M(a',a'')$ indicates a selective measurement in which systems are accepted only in the state a'' and emerge in the state a'. The measurement process $M(a')$ is the special case for which no change of state occurs,

$$M(a') = M(a',a').$$

The properties of successive measurements of the type $M(a',a'')$ are symbolized by

$$M(a',a'')M(a''',a^{\mathrm{iv}}) = \delta(a'',a''')M(a',a^{\mathrm{iv}}), \tag{4}$$

for, if $a'' \neq a'''$, the second stage of the compound apparatus accepts none of the systems that emerge from the first stage, while if $a'' = a'''$, all such systems enter the second stage and the compound measurement serves to select systems in the state a^{iv} and produce them in the state a'. Note that if the two stages are reversed, we have

$$M(a''',a^{\mathrm{iv}})M(a',a'') = \delta(a',a^{\mathrm{iv}})M(a''',a''),$$

which differs in general from equation (4). Hence the multiplication of measurement symbols is noncommutative.

The physical quantities contained in one complete set A do not comprise the totality of physical attributes of the system. One can form other complete sets, $B, C, \ldots$, which are mutually incompatible, and for each choice of noninterfering physical characteristics there is a set of selective measurements referring to systems in the appropriate states, $M(b',b'')$,$M(c',c'') \ldots$. The most general selective measurement involves two incompatible sets of properties. We symbolize by $M(a',b')$ the measurement process that rejects all impinging systems except those in the state b', and permits only systems in the state a' to emerge from the appara-

tus. The compound measurement $M(a',b')M(c',d')$ serves to select systems in the state d' and produce them in the state a', which is a selective measurement of the type $M(a',d')$. But, in addition, the first stage supplies systems in the state c' while the second stage accepts only systems in the state b'. The examples of compound measurements that we have already considered involve the passage of all systems or no systems between the two stages, as represented by the multiplicative numbers 1 or 0. More generally, measurements of properties B, performed on a system in a state c' that refers to properties incompatible with B, will yield a statistical distribution of the possible values. Hence, only a determinate fraction of the systems emerging from the first stage will be accepted by the second stage. We express this by the general multiplication law

$$M(a',b')M(c',d') = \langle b'|c'\rangle M(a',d'), \tag{5}$$

where $\langle b'|c'\rangle$ is a number characterizing the statistical relation between the states b' and c'. In particular,

$$\langle a'|a''\rangle = \delta(a',a'').$$

Special examples of (5) are

$$M(a')M(b',c') = \langle a'|b'\rangle M(a',c')$$

and

$$M(a',b')M(c') = \langle b'|c'\rangle M(a',c').$$

We infer from the fundamental measurement symbol property (3) that

$$\sum_{a'}\langle a'|b'\rangle M(a',c') = \sum_{a'} M(a')M(b',c') = M(b',c')$$

and similarly

$$\sum_{c'}\langle b'|c'\rangle M(a',c') = M(a',b'),$$

which shows that measurement symbols of one type can be expressed as a linear combination of the measurement symbols of another type. The general relation is

$$M(c',d') = \sum_{a'b'} M(a')M(c',d')M(b') = \sum_{a'b'}\langle a'|c'\rangle\langle d'|b'\rangle M(a',b'). \tag{6}$$

From its role in effecting such connections, the totality of numbers $\langle a'|b'\rangle$ is called the transformation function relating the a- and b-descriptions, where the phrase "a-description" signifies the description of a system in terms of the states produced by selective measurements of the complete set of compatible physical quantities A.

A fundamental composition property of transformation functions is obtained on comparing

$$\sum_{b'} M(a')M(b')M(c') = \sum_{b'}\langle a'|b'\rangle\langle b'|c'\rangle M(a',c')$$

with

$$M(a')\Big(\sum_{b'}M(b')\Big)M(c') = M(a')M(c') = \langle a'|c'\rangle M(a',c'),$$

namely

$$\sum_{b'}\langle a'|b'\rangle\langle b'|c'\rangle = \langle a'|c'\rangle.$$

On identifying the a- and c-descriptions this becomes

$$\sum_{b'}\langle a'|b'\rangle\langle b'|a''\rangle = \delta(a',a'')$$

and similarly

$$\sum_{a'}\langle b'|a'\rangle\langle a'|b''\rangle = \delta(b',b'').$$

As a consequence, we observe that

$$\sum_{a'}\sum_{b'}\langle a'|b'\rangle\langle b'|a'\rangle = \sum_{a'}1$$
$$=\sum_{b'}\sum_{a'}\langle b'|a'\rangle\langle a'|b'\rangle = \sum_{b'}1,$$

which means that N, the total number of states obtained in a complete measurement, is independent of the particular choice of compatible physical quantities that are measured. Hence the total number of measurement symbols of any specified type is N^2. Arbitrary numerical multiples of measurement symbols in additive combination thus form the elements of a linear algebra of dimensionality N^2—the algebra of measurement. The elements of the measurement algebra are called operators.

The number $\langle a'|b'\rangle$ can be regarded as a linear numerical function of the operator $M(b',a')$. We call this linear correspondence between operators and numbers the trace,

$$\langle a'|b'\rangle = trM(b',a'), \tag{7}$$

and observe from the general linear relation (6) that

$$trM(c',d') = \sum_{a'b'}\langle a'|c'\rangle\langle d'|b'\rangle trM(a',b')$$
$$= \sum_{a'b'}\langle d'|b'\rangle\langle b'|a'\rangle\langle a'|c'\rangle$$
$$= \langle d'|c'\rangle,$$

which verifies the consistency of the definition (7). In particular,

$$trM(a',a'') = \delta(a',a'')$$
$$trM(a') = 1.$$

The trace of a measurement symbol product is

$$trM(a',b')M(c',d') = \langle b'|c'\rangle trM(a',d') = \langle b'|c'\rangle\langle d'|a'\rangle, \tag{8}$$

which can be compared with

$$trM(c',d')M(a',b') = \langle d'|a'\rangle trM(c',b') = \langle d'|a'\rangle\langle b'|c'\rangle.$$

Hence, despite the noncommutativity of multiplication, the trace of a product of two factors is independent of the multiplication order. This applies to any two elements X, Y, of the measurement algebra,

$$trXY = trYX.$$

A special example of (8) is

$$trM(a')M(b') = \langle a'|b'\rangle\langle b'|a'\rangle \tag{9}$$

It should be observed that the general multiplication law and the definition of the trace are preserved if we make the substitutions

$$\begin{aligned} M(a',b') &\rightarrow \lambda(a')^{-1}M(a',b')\lambda(b') \\ \langle a'|b'\rangle &\rightarrow \lambda(a')\langle a'|b'\rangle\lambda(b')^{-1}, \end{aligned} \tag{10}$$

where the numbers $\lambda(a')$ and $\lambda(b')$ can be given arbitrary nonzero values. The elementary measurement symbols $M(a')$ and the transformation function $\langle a'|a''\rangle$ are left unaltered. In view of this arbitrariness, a transformation function $\langle a'|b'\rangle$ cannot, of itself, possess a direct physical interpretation but must enter in some combination that remains invariant under the substitution (10).

The appropriate basis for the statistical interpretation of the transformation function can be inferred by a consideration of the sequence of selective measurements $M(b')M(a')M(b')$, which differs from $M(b')$ in virtue of the disturbance attendant upon the intermediate A-measurement. Only a fraction of the systems selected in the initial B-measurement is transmitted through the complete apparatus. Correspondingly, we have the symbolic equation

$$M(b')M(a')M(b') = p(a',b')M(b'),$$

where the number

$$p(a',b') = \langle a'|b'\rangle\langle b'|a'\rangle \tag{11}$$

is invariant under the transformation (10). If we perform an A-measurement that does not distinguish between two (or more) states, there is a related additivity of the numbers $p(a',b')$,

$$M(b')(M(a') + M(a''))M(b') = (p(a',b') + p(a'',b'))M(b)',$$

and, for the A-measurement that does not distinguish among any of the states, there appears

$$M(b')(\sum_{a'}M(a'))M(b') = M(b'),$$

whence

$$\sum_{a'}p(a',b') = 1.$$

These properties qualify $p(a',b')$ for the role of the probability that one observes the state a' in a measurement performed on a system known to be in the state b'.

But a probability is a real, nonnegative number. Hence we shall impose an admissible restriction on the numbers appearing in the measurement algebra, by requiring that $\langle a'|b'\rangle$ and $\langle b'|a'\rangle$ form a pair of complex conjugate numbers.[2]

$$\langle b'|a'\rangle = \langle a'|b'\rangle^*, \tag{12}$$

for then

$$p(a',b') = |\langle a'|b'\rangle|^2 \geq 0.$$

To maintain the complex conjugate relation (12), the numbers $\lambda(a')$ of (10) must obey

$$\lambda(a')^* = \lambda(a')^{-1},$$

and therefore have the form

$$\lambda(a') = e^{i\varphi(a')}$$

in which the phases $\varphi(a')$ can assume arbitrary real values.

Another satisfactory aspect of the probability formula (11) is the symmetry property

$$p(a',b') = p(b',a').$$

Let us recall the arbitrary convention that accompanies the interpretation of the measurement symbols and their products—the order of events is read from right to left (sinistrally). But any measurement symbol equation is equally valid if interpreted in the opposite sense (dextrally), and no physical result should depend upon which convention is employed. On introducing the dextral interpretation, $\langle a'|b'\rangle$ acquires the meaning possessed by $\langle b'|a'\rangle$ with the sinistral convention. We conclude that the probability connecting states a' and b' in given sequence must be constructed symmetrically from $\langle a'|b'\rangle$ and $\langle b'|a'\rangle$. The introduction of the opposite convention for measurement symbols will be termed the adjoint operation, and is indicated by†. Thus,

$$M(a',b')\dagger = M(b',a')$$

and

$$M(a',a'')\dagger = M(a'',a').$$

In particular,

$$M(a')\dagger = M(a'),$$

which characterizes $M(a')$ as a self-adjoint or Hermitian operator. For measurement symbol products we have

$$\begin{aligned}(M(a',b')M(c',d'))\dagger &= M(d',c')M(b',a') \\ &= M(c',d')\dagger M(a',b')\dagger,\end{aligned}$$

or equivalently,

$$\begin{aligned}(\langle b'|c'\rangle M(a',d'))\dagger &= \langle c'|b'\rangle M(d',a') \\ &= \langle b'|c'\rangle^* M(a',d')\dagger.\end{aligned}$$

The significance of addition is uninfluenced by the adjoint procedure, which permits us to extend these properties to all elements of the measurement algebra:

$$(X + Y)\dagger = X\dagger + Y\dagger, (XY)\dagger = Y\dagger X\dagger, (\lambda X)\dagger = \lambda^* X\dagger,$$

in which λ is an arbitrary number.

The use of complex numbers in the measurement algebra implies the existence of a dual algebra in which all numbers are replaced by the complex conjugate numbers. No physical result can depend upon which algebra is employed. If the operators of the dual algebra are written X^*, the correspondence between the two algebras is governed by the laws

$$(X + Y)^* = X^* + Y^*, (XY)^* = X^*Y^*, (\lambda X)^* = \lambda^* X^*.$$

The formation of the adjoint within the complex conjugate algebra is called transposition,

$$X^\tau = X^*\dagger = X\dagger^*.$$

It has the algebraic properties

$$(X + Y)^\tau = X^\tau + Y^\tau, (XY)^\tau = Y^\tau X^\tau, (\lambda X)^\tau = \lambda X^\tau.$$

The measurement symbols of a given description provide a basis for the representation of an arbitrary operator by N^2 numbers, and the abstract properties of operators are realized by the combinatorial laws of these arrays of numbers, which are those of matrices. Thus

$$X = \sum_{a'a''} \langle a' | X | a'' \rangle M(a',a'')$$

defines the matrix of X in the a-description or a-representation, and the product

$$\begin{aligned} XY &= \Sigma \langle a' | X | a'' \rangle M(a',a'') \Sigma \langle a^{\mathrm{iv}} | Y | a''' \rangle M(a^{\mathrm{iv}},a''') \\ &= \Sigma \langle a' | X | a'' \rangle \delta(a'',a^{\mathrm{iv}}) \langle a^{\mathrm{iv}} | Y | a''' \rangle M(a',a''') \end{aligned}$$

shows that

$$\langle a' | XY | a''' \rangle = \sum_{a''} \langle a' | X | a'' \rangle \langle a'' | Y | a''' \rangle.$$

The elements of the matrix that represents X can be expressed as

$$\langle a' | X | a'' \rangle = trXM(a'',a'),$$

and in particular

$$\langle a' | X | a' \rangle = trXM(a').$$

The sum of the diagonal elements of the matrix is the trace of the operator. The corresponding basis in the dual algebra is $M(a',a'')^*$, and the matrices that represent X^* and X^τ are the complex conjugate and transpose, respectively, of the matrix representing X. The operator $X\dagger = X^{\tau *}$, an element of the same algebra as X, is represented by the transposed, complex conjugate, or adjoint matrix.

The matrix of X is the mixed ab-representation is defined by

$$X = \sum_{a'b'} \langle a' | X | b' \rangle M(a',b')$$

where

$$\langle a'|X|b'\rangle = trXM(b',a').$$

The rule of multiplication for matrices in mixed representations is

$$\langle a'|XY|c'\rangle = \sum_{b'}\langle a'|X|b'\rangle\langle b'|Y|c'\rangle.$$

On placing $X = Y = 1$ we encounter the composition property of transformation functions, since

$$\begin{aligned}\langle a'|1|b'\rangle &= trM(b',a')\\ &= \langle a'|b'\rangle.\end{aligned}$$

If we set X or Y equal to 1, we obtain examples of the connection between the matrices of a given operator in various representations. The general result can be derived from the linear relations among measurement symbols. Thus,

$$\begin{aligned}\langle a'|X|d'\rangle &= trXM(d',a')\\ &= trX\sum_{b'c'}\langle c'|d'\rangle\langle a'|b'\rangle M(c',b')\\ &= \sum_{b'c'}\langle a'|b'\rangle\langle b'|X|c'\rangle\langle c'|d'\rangle.\end{aligned}$$

The adjoint of an operator X, displayed in the mixed ab-basis, appears in the ba-basis with the matrix

$$\langle b'|X\dagger|a'\rangle = \langle a'|X|b'\rangle^*.$$

As an application of mixed representations, we present an operator equivalent of the fundamental properties of transformation functions:

$$\begin{aligned}\sum_{b'}\langle a'|b'\rangle\langle b'|c'\rangle &= \langle a'|c'\rangle\\ \langle a'|b'\rangle^* &= \langle b'|a'\rangle,\end{aligned}$$

which is achieved by a differential characterization of the transformation functions. If $\delta\langle a'|b'\rangle$ and $\delta\langle b'|c'\rangle$ are any conceivable infinitesimal alteration of the corresponding transformation functions, the implied variation of $\langle a'|c'\rangle$ is

$$\delta\langle a'|c'\rangle = \sum_{b'}[\delta\langle a'|b'\rangle\langle b'|c'\rangle + \langle a'|b'\rangle\delta\langle b'|c'\rangle], \tag{13}$$

and also

$$\delta\langle a'|b'\rangle^* = \delta\langle b'|a'\rangle.$$

One can regard the array of numbers $\delta\langle a'|b'\rangle$ as the matrix of an operator in the ab-representation. We therefore write

$$\delta\langle a'|b'\rangle = i\langle a'|\delta W_{ab}|b'\rangle,$$

which is the definition of an infinitesimal operator δW_{ab}. If infinitesimal operators δW_{bc} and δW_{ac} are defined similarly, the differential property (13) becomes the matrix equation

$$\langle a'|\delta W_{ac}|c'\rangle = \sum_{b'}[\langle a'|\delta W_{ab}|b'\rangle\langle b'|c'\rangle + \langle a'|b'\rangle\langle b'|\delta W_{bc}|c'\rangle],$$

from which we infer the operator equation

$$\delta W_{ac} = \delta W_{ab} + \delta W_{bc}. \tag{14}$$

Thus the multiplicative composition law of transformation functions is expressed by an additive composition law for the infinitesimal operators δW.

On identifying the a- and b-descriptions in (14), we learn that

$$\delta W_{aa} = 0$$

or

$$\delta\langle a' | a'' \rangle = 0,$$

which expresses the fixed numerical values of the transformation function

$$\langle a' | a'' \rangle = \delta(a',a'').$$

Indeed, the latter is not an independent condition on transformation functions but is implied by the composition property and the requirement that transformation functions, as matrices, be nonsingular. If we identify the a- and c-descriptions we are informed that

$$\delta W_{ba} = -\delta W_{ab}.$$

Now

$$\begin{aligned} \delta\langle a' | b' \rangle^* &= -i\langle a' | \delta W_{ab} | b' \rangle^* \\ &= -i\langle b' | \delta W_{ab}\dagger | a' \rangle, \end{aligned}$$

which must equal

$$\delta\langle b' | a' \rangle = i\langle b' | \delta W_{ba} | a' \rangle,$$

and therefore

$$\delta W_{ab}\dagger = -\delta W_{ba} = \delta W_{ab}.$$

The complex conjugate property of transformation functions is thus expressed by the statement that the infinitesimal operators δW are Hermitian.

The expectation value of property A for systems in the state b' is the average of the possible values of A, weighted by the probabilities of occurrence that are characteristic of state b'. On using (9) to write the probability formula as

$$p(a',b') = trM(a')M(b'),$$

the expectation value becomes

$$\begin{aligned} \langle A \rangle_{b'} &= \sum_{a'} a' p(a',b') = trAM(b') \\ &= \langle b' | A | b' \rangle, \end{aligned}$$

where the operator A is

$$A = \sum_{a'} a' M(a').$$

The correspondence thus obtained between operators and physical quantities is such that a function $f(A)$ of the property A is assigned the operator $f(A)$, and the opera-

tors associated with a complete set of compatible physical quantities form a complete set of commuting Hermitian operators. In particular, the function of A that exhibits the value unity in the state a', and zero otherwise, is characterized by the operator $M(a')$.

The physical operation symbolized by $M(a')$ involves the functioning of an apparatus capable of separating an ensemble into subensembles that are distinguished by the various values of a', together with the act of selecting one subensemble and rejecting the others. The measurement process prior to the stage of selection, which we call a nonselective measurement, will now be considered for the purpose of finding its symbolic counterpart. It is useful to recognize a general quantitative interpretation attached to the measurement symbols. Let a system in the state c' be subjected to the selective $M(b')$ measurement and then to an A-measurement. The probability that the system will exhibit the value b' and then a', for the respective properties, is given by

$$\begin{aligned} p(a',b',c') &= p(a',b')p(b',c') = |\langle a'|b'\rangle\langle b'|c'\rangle|^2 \\ &= |\langle a'|M(b')|c'\rangle|^2. \end{aligned}$$

If, in contrast, the intermediate B-measurement accepts all systems without discrimination, which is equivalent to performing no B-measurement, the relevant probability is

$$\begin{aligned} p(a',1,c') &= |\langle a'|c'\rangle|^2 \\ &= |\langle a'|\sum_{b'} M(b')|c'\rangle|^2. \end{aligned}$$

There are examples of the relation between the symbol of any selective measurement and a corresponding probability,

$$p(a',\ ,c') = |\langle a'|M|c'\rangle|^2.$$

Now let the intervening measurement be nonselective, which is to say that the apparatus functions but no selection of systems is performed. Accordingly,

$$\begin{aligned} p(a',b,c') &= \sum_{b'} p(a',b')p(b',c') \\ &= \sum_{b'} |\langle a'|M(b')|c'\rangle|^2 \end{aligned}$$

which differs from

$$p(a',1,c') = |\sum_{b'}\langle a'|M(b')|c'\rangle|^2$$

by the absence of interference terms between different b' states. This indicates that the symbol to be associated with the nonselective B-measurement is

$$M_b = \sum_{b'} e^{i\varphi_{b'}} M(b')$$

where the real phases $\varphi_{b'}$ are independent, randomly distributed quantities. The uncontrollable nature of the disturbance produced by a measurement thus finds its mathematical expression in these random phase factors. Since a nonselective measurement does not discard systems we must have

$$\sum_{a'} p(a',b,c') = 1$$

which corresponds to the unitary property of the M_b operators,

$$M_b\dagger M_b = M_b M_b\dagger = 1.$$

It should also be noted that, within this probability context, the symbols of the elementary selective measurements are derived from the nonselective symbol by replacing all but one of the phases by positive infinite imaginary numbers, which is an absorptive description of the process of rejecting subensembles.

The general probability statement for successive measurements is

$$p(a',b',\ldots s',t') = |\langle a'|M(b')\ldots M(s')|t'\rangle|^2$$

which is applicable to any type of observation by inserting the appropriate measurement symbol. Other versions are

$$p(a',\ldots t') = \langle t'|(M(a')\ldots M(s'))\dagger(M(a')\ldots M(s'))|t'\rangle$$

and

$$p(a',\ldots t') = tr(M(a')\ldots M(t'))\dagger(M(a')\ldots M(t')),$$

each of which can also be extended to all types of selective measurements, and to nonselective measurements (the adjoint form is essential here). The expectation value construction shows that a quantity which equals unity if the properties A, $B,\ldots S$ successively exhibit, in the sinistral sense, the values a', b', $\ldots s'$, and is zero otherwise, is represented by the Hermitian[3] operator $(M(a')\ldots M(s'))\dagger$-$(M(a')\ldots M(s'))$.

Measurement is a dynamical process, and yet the only time concept that has been used is the primitive relationship of order. A detailed formulation of quantum dynamics must satisfy the consistency requirement that its description of the interactions that constitute measurement reproduces the symbolic characterizations that have emerged at this elementary stage. Such considerations make explicit reference to the fact that all measurement of atomic phenomena ultimately involves the amplification of microscopic effects to the level of macroscopic observation.

Further analysis of the measurement algebra leads to a geometry associated with the states of systems.

[1] This development has been presented in numerous lecture series since 1951, but is heretofore unpublished.

[2] Here we bypass the question of the utility of the real number field. According to a comment in THESE PROCEEDINGS, **44**, 223 (1958), the appearance of complex numbers, or their real equivalents, may be an aspect of the fundamental matter-antimatter duality, which can hardly be discussed at this stage.

[3] Compare P. A. M. Dirac, *Rev. Mod. Phys*, **17**, 195 (1945), where non-Hermitian operators and complex "probabilities" are introduced.

ACKNOWLEDGMENT OF PRIORITY

The author wishes to thank H. D. Brunk for bringing to his attention that inequality (4) contained in "On Inequalities of Szegö and Bellman," these PROCEEDINGS, 45, 230–231 (1959), can be obtained from corollary (3) of H. D. Brunk, "Inequality for Convex Functions," *Proc. Amer. Math. Soc.*, 7, 817–824 (1956).

INGRAM OLKIN

ERRATA

In my paper "A Theorem on Convex Bodies and Applications to Banach Spaces," these PROCEEDINGS, **45**, 223–226 (1959), I wish to substitute the following for the first paragraph on p. 225:

Let $^1/_3 > \epsilon > 0$ and assume that no E^k intersects the boundary of C on a set contained in $(1 - \epsilon)\rho \leq \|x\| \leq (1 + \epsilon)\rho$. Let $V'[U']$ be the projection (from the origin) on S_n of the boundary points of C lying outside $(1 + \epsilon)\rho\, S_n$ [inside $(1 - \epsilon)\rho\, S_n$]. Since, by assumption, $\nu_{n,k}(V' \cup U') = 1$ it follows that $\nu_{n,k}(V') \geq {}^1/_2$ or $\nu_{n,k}(U') \geq {}^1/_2$; assume, for definiteness, that the first inequality holds. It then follows from (9) and lemmas 1 and 3 that $\nu_{n,1}(V'_{\epsilon/3\rho}) > {}^2/_5$ for n sufficiently large. Let $\bar{V}[\bar{U}]$ be the set of boundary points of C whose distance from the origin is $\geq (1 + \epsilon/2)\rho$ $[\leq \rho]$ and $\bar{V}'[\bar{U}']$ be its projection on S_n. Since, by Lemma 2, $\bar{V}' \supset V'_{\epsilon/3\rho}$ we have $\nu_{n,1}(\bar{V}') > {}^2/_5$; also, by (8), $\nu_{n,1}(\bar{U}') \geq {}^1/_2$. If W denotes the set of E^k meeting both $\bar{V}$ and $\bar{U}$ it is easily seen from (4) that $\mu_{n,k}(W) \geq 1 - ({}^1/_2)^2 - ({}^2/_5)^2 > {}^1/_3$ for $k \geq 2$. From all this it follows that $\nu_{n,k}(\bar{U}') + \nu_{n,k}(\bar{V}') > {}^4/_3$. Applying again our lemmas we find that, for sufficiently large n, the sets $\bar{U}'_{\epsilon/3\rho}$ and $\bar{V}'_{\epsilon/3\rho}$ are disjoint while their $\nu_{n,1}$ measures add up to more than 1. This contradiction proves Theorem 1 under assumption (7).

ARYEH DVORETZKY

INFORMATION TO CONTRIBUTORS

THE PROCEEDINGS is the official organ of the NATIONAL ACADEMY OF SCIENCES and of the NATIONAL RESEARCH COUNCIL for the publication of brief accounts of important current researches of members of the ACADEMY and of the COUNCIL and of other American investigators. The PROCEEDINGS will aim especially to secure prompt publication of original announcements of discoveries and wide circulation of the results of American research among investigators in other countries and in all branches of science.

ARTICLES should be brief. The viewpoint should be comprehensive in giving the relation of the paper to previous publications of the author or of others and in exhibiting, where practicable, the significance of the work for other branches of science. Elaborate technical details of the work and long tables of data should be avoided, but authors should be precise in making clear the new results and should give some record of the methods and data upon which they are based.

MANUSCRIPTS should be prepared with a current number of the PROCEEDINGS as a model in matters of form, and should be typewritten in duplicate with double spacing, the author retaining one copy. Illustrations should be confined to text figures of simple character, though more elaborate illustration may be allowed in special instances to authors willing to pay for their preparation and insertion. Particular attention should be given to arranging tabular matter in a simple and concise manner.

MANUSCRIPTS will be accepted only from members of the Academy who will assume responsibility for the propriety and scientific standards of the paper and for any printing costs in excess of those allowed them by the Academy. Manuscripts should be addressed to THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, 2101 Constitution Ave., Washington 25, D. C.

GALLEY PROOF will be sent, with the understanding that charges for author's corrections shall be billed to him. Authors should therefore make final revisions on the typewritten manuscripts. Original artwork or figures will be returned if requested when the manuscript is submitted. Because of time limitations, page proofs will not be sent.

REPRINTS should be ordered when proof is returned, on the reprint order form which will be enclosed with the proof. They will be furnished at cost, approximately as follows:

SCHEDULE OF REPRINT RATES (POSTAGE NOT INCLUDED)

Quantity	*up to 4 pp.*	*up to 8 pp.*	*up to 12 pp.*	*Standard Covers*
50	$ 7.85	$15.10	$22.30	$6.90
100	8.95	17.30	25.60	8.40
150	10.10	19.50	28.95	9.95
add'l 100's	2.20	4.40	6.60	3.05

CONTENTS

		Page
BIOCHEMISTRY.—COORDINATE REPRESSION OF THE SYNTHESIS OF FOUR HISTIDINE BIOSYNTHETIC ENZYMES BY HISTIDINE	*By Bruce N. Ames and Barbara Garry*	1453
BIOCHEMISTRY.—AMINO ACID ACTIVATION AND TRANSFER TO RIBONUCLEIC ACIDS IN THE CELL NUCLEUS	*By J. W. Hopkins*	1461
BIOCHEMISTRY.—A GENERAL METHOD FOR THE LABELING OF THE ACTIVE SITE OF ANTIBODIES AND ENZYMES	*By Marian Elliott Koshland, Frieda Engleberger, and D. E. Koshland, Jr.*	1470
BIOCHEMISTRY.—ON THE METABOLIC BREAKDOWN OF HEMOGLOBIN AND THE ELECTRONIC STRUCTURE OF THE BILE PIGMENTS	*By Bernard Pullman and Anne-Marie Perault*	1476
BIOCHEMISTRY.—A STATISTICAL ANALYSIS OF DEOXYRIBONUCLEIC ACID DISTRIBUTION IN DENSITY GRADIENT CENTRIFUGATION	*By Noboru Sueoka*	1480
BIOCHEMISTRY.—THE BIOSYNTHESIS OF *p*-AMINOBENZOIC ACID	*By B. Weiss and P. R. Srinivasan*	1491
GENETICS.—GLUCOSE INHIBITION AND THE DIAUXIE PHENOMENON	*By Ellis Englesberg*	1494
GENETICS.—MUTATIONAL HETEROZYGOTES IN BACTERIOPHAGES	*By David Pratt and Gunther S. Stent*	1507
GENETICS.—NON-RANDOM GENE DISTRIBUTION AMONG TOMATO CHROMOSOMES	*By Charles M. Rick*	1515
MATHEMATICS.—ON THE RADICAL OF A POSITIVE SEMIRING	*By Samuel Bourne*	1519
MATHEMATICS.—ON SUBMANIFOLDS OF CERTAIN FUNCTION SPACES	*By James Eells, Jr.*	1520
MATHEMATICS.—EXTERIOR DIFFERENTIAL SYSTEMS AND ELLIPTIC EQUATIONS	*By Bruce L. Reinhart and Shlomo Sternberg*	1522
MATHEMATICS.—POLYNOMIALS OF BEST APPROXIMATION ON AN INTERVAL	*By J. L. Walsh and T. S. Motzkin*	1523
MATHEMATICS.—NOTE ON INVARIANCE OF DEGREE OF POLYNOMIAL AND TRIGONOMETRIC APPROXIMATION UNDER CHANGE OF INDEPENDENT VARIABLE	*By J. L. Walsh*	1528
MICROBIOLOGY.—BIOASSAY OF ORGANIC MICRONUTRIENTS IN THE SEA	*By William L. Belser*	1533
PHYSICS.—THE ALGEBRA OF MICROSCOPIC MEASUREMENT	*By Julian Schwinger*	1542
ACKNOWLEDGMENT.—ON INEQUALITIES OF SZEGÖ AND BELLMAN	*By Ingram Olkin*	1553
ERRATA.—A THEOREM ON CONVEX BODIES AND APPLICATIONS TO BANACH SPACES	*By Aryeh Dvoretzky*	1554